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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM

No. 22

PRECURSOR INCORPORATION EXPERIMENTS IN PAPAVER ALKALOID BIOSYNTHESIS

I. PAPAVER BRACTEATUM LINDL.

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GE. 76-5110

PRECURSOR INCORPORATION EXPERIMENTS IN PAPAVER ALKALOID BIOSYNTHESIS

I. PAPAVER BRACTEATUM LINDL. [1]

It has been known for several years that different species or strains of the fungus Penicillium are able to incorporate precursors and that the yield of penicillin is increased when suitable precursors are added to the growth medium (1). It has also been shown that weed-killers of the hormone type are able to penetrate into the aerial parts of plants when solutions of such compounds are sprayed on the fields (2). These observations led us to investigate whether the same principle could be used for the introduction into alkaloid-producing plants of nitrogenous compounds, such as amino acids dissolved in a surface active agent. If so, we assumed that the compounds would enter the metabolic pathway of the plants and act as precursors in the biosynthesis of alkaloids.

Phenylalanine, tyrosine, glycine and urea, all uniformly labelled with ^{14}C, were chosen as the precursors to be studied. The experiments were carried out using Papaver bracteatum Lindl. (Arya II) as test organism. After exposure of the precursors to the plant surface for a certain time, the plants were extracted with ethanolic ammonia and the low molecular weight radioactive compounds separated by TLC and located by radioautography. The radioactivity of the unconverted isotopes (A), of the radioactive metabolites (B), and of the radioactive thebaine (C) were measured by liquid scintillation counting and the results presented in a diagram (Table 1).

1/ The present study forms part of the international research programme organized by the United Nations Narcotics Laboratory at the request of the Commission on Narcotic Drugs. The objective of this programme is to increase the yield of phenanthrene alkaloids per unit of cultivated area in order to facilitate control and to ensure adequate supplies of codeine for medical purposes.

EXPERIMENTAL PROCEDURE

Plant material. For the experiments, we used Papaver bracteatum Lindl. (Arya II), grown in the Botanical Garden, University of Oslo, Tøyen, from seeds kindly provided by Dr. O.J. Braenden, U.N. Narcotics Laboratory, Geneva. At the start of the experiments, the plants had shed their petals, but the capsules were still fresh and green (Figures 1 and 2).

Labelled precursors. Uniformly ^{14}C labelled compounds were used (250 μ Ci of each): L-phenylalanine, L-tyrosine, L-glycine as hydrochlorides and urea, all supplied by New England Nuclear. Before use, the solutions of the amino acids were neutralized with sodium hydrogen carbonate, freeze-dried and dissolved in 1.5 ml of a 1% solution of the surfactant: MON 0011, Monsanto Company, St. Louis, Miss., U.S.A., kindly supplied by Dr. Kåre Lund-Høye, The Agricultural University of Norway, Ås. The urea was freed from ethanol by evaporation and then dissolved in 1 ml of the same surfactant solution as above.

Application of labelled precursors. Each of the precursors was applied to separate plants. The solutions of phenylalanine, tyrosine, and urea were applied onto the upper part of the stem and to the four upper leaves. Glycine was applied onto the capsule. After application of the isotopes, the plants were covered with plastic bags having the corners cut off. This was done to prevent rain from washing the isotopes off the plants and to prevent condensation of water inside the bags (Figure 2). The plants were left photosynthesizing from August 8 to September 3, 1975. During this period the weather was warm and sunny in the Oslo area, for which reason the plants were irrigated carefully a few times. After harvesting, the plants were scanned for radioactivity using a "Nardeux" Geiger-Muller-counter. Radioactive material appeared to be present in all parts of the plants except the root. The following parts of the plant were analysed for their content of radioactive material: capsule, stem, leaves and root-stem transition.

Extraction of the plants. The whole capsule, parts of stem, leaves and root-stem transition from the four treated plants were extracted separately with ethanolic ammonia (5%) at about 70°C. After filtration and concentration, the extracts were subjected to TLC using systems 1 and 2.

TLC plates. Polygram Sil G/UV 254 from Macherey-Nagel & Co. (20 x 20 cm). The compounds were visualized by means of UV-light.

Thin layer chromatography (TLC) was carried out in the following solvent systems: 1. toluene: acetone: ethanol: 25% ammonia (45:45:7:3) vol/vol; 2. ethyl acetate: methanol: 25% ammonia (85:10:5) vol/vol, freshly prepared.

Radioautography. The TLC plates were exposed to Kodak RP X-omat Medical X-ray film RP54 for about two weeks before development. The radioactive compounds were thus located on the TLC plates (Figures 3 and 4) and the appropriate areas cut out and subjected to liquid scintillation counting.

Liquid scintillation counting was carried out in a Mark II liquid scintillation system, Nuclear - Chicago. As scintillant the following solution was used: PPO 5 g., Dimethyl POPOP 0.3 g., Toluene 1 l.

RESULTS AND DISCUSSION

The experiments show that phenylalanine, tyrosine, glycine and urea are able to penetrate Papaver bracteatum when dissolved in a 1% aqueous solution of a certain surfactant. From Table 1 it can be seen that a relatively high percentage of the labelled precursors has been converted into radioactive metabolites extractable with ethanolic ammonia.

As expected, radioactive thebaine was found in the plants treated with phenylalanine and tyrosine. However, it was also found that glycine and urea gave rise to radioactive thebaine (Figure 3, a-d, and Table 1). The figures show that several radioactive metabolites were formed, and also that very little radioactivity was present in the root-stem transitions and in the leaves of the four plants. No radioactive compounds were found in the roots. The non-metabolized amino acids and urea will remain at the place of application on the TLC-plates as they do not move in the solvent systems employed.

In order to avoid injury to the plants before they had reached a certain degree of maturity, the labelled precursors were applied at a late stage of development of the poppy plants. Another reason for the time chosen was that

the alkaloid biosynthesis was assumed to be most active during the period of development when the latex production of the plant was at its highest.

TABLE 1

(See Figure 3, a-d, and Figure 4)

	PHENYLALANINE		TYROSINE		GLYCINE		UREA	
	$\frac{Bx100}{A+B}$	$\frac{Cx100}{A+B}$	$\frac{Bx100}{A+B}$	$\frac{Cx100}{A+B}$	$\frac{Bx100}{A+B}$	$\frac{Cx100}{A+B}$	$\frac{Bx100}{A+B}$	$\frac{Cx100}{A+B}$
Capsule	57.4	13.6	25.8	1.2	50.2	3.7	56.3	2.4
Leaves	43.8	3.8	41.4	1.1	12.6	0.3	72.8	3.0
Stem	8.0	1.2	28.6	0.9	13.7	0.8	34.5	0.8
Root-stem transition	54.0	3.4	36.8	5.6	24.6	0.9	32.5	1.6

A: C/pm of unconverted precursor remaining at the starting line of the chromatogram.

B: C/pm of radioactive metabolites derived from the precursor.

C: C/pm of thebaine.

The figures under the headings $\frac{Bx100}{A+B}$ show the percentage of radioactive metabolites, and $\frac{Cx100}{A+B}$ the corresponding percentage of radioactive thebaine found in the extracts.

Although preliminary, the present experiments show clearly that amino acids and, even more important, urea applied to leaf and stem surfaces of P. bracteatum are absorbed by the plant and utilized in part in alkaloid biosynthesis.

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2. Ashton, Floid M., and Alden S. Crafts: Mode and Action of Herbicides. Wiley Interscience Publications, 1973.

Figure 1 - Papaver bracteatum

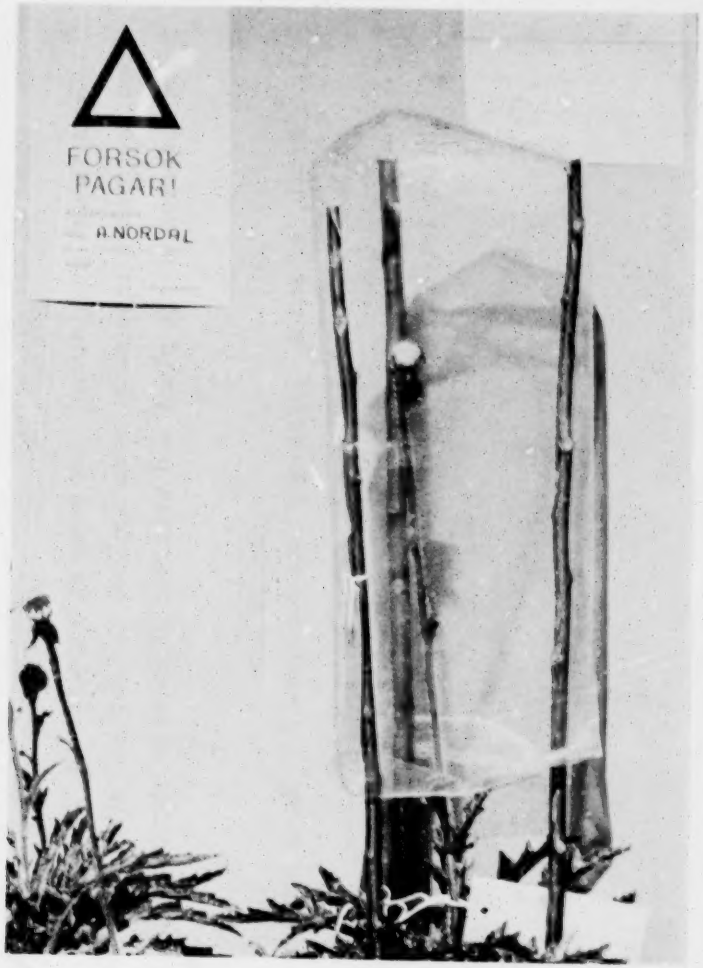

Figure 2 - Running experiment

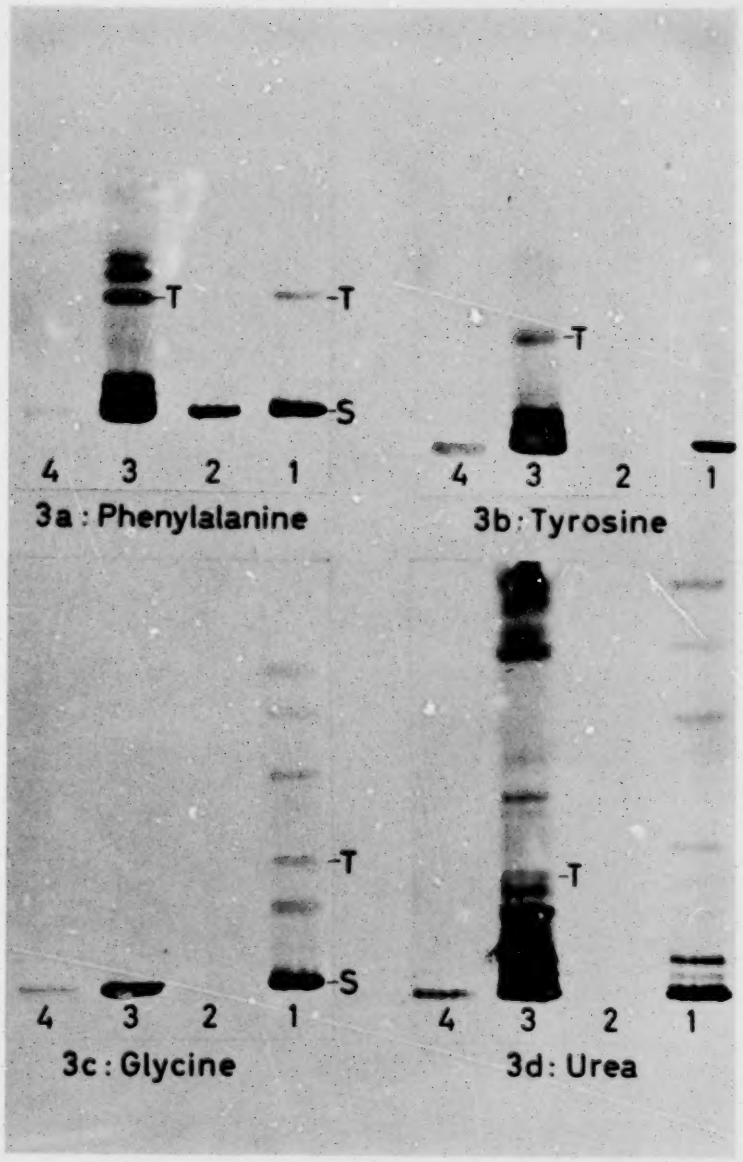

Figure 3 - Incorporation of different precursors into Papaver bracteatum. The numbers 1, 2, 3 and 4 show radioautograms of TLC plates derived from extracts of capsules, leaves and stems, and from the root-stem transition of the plants. "S" signifies the starting line and "T" the location of thebaine on the chromatograms.

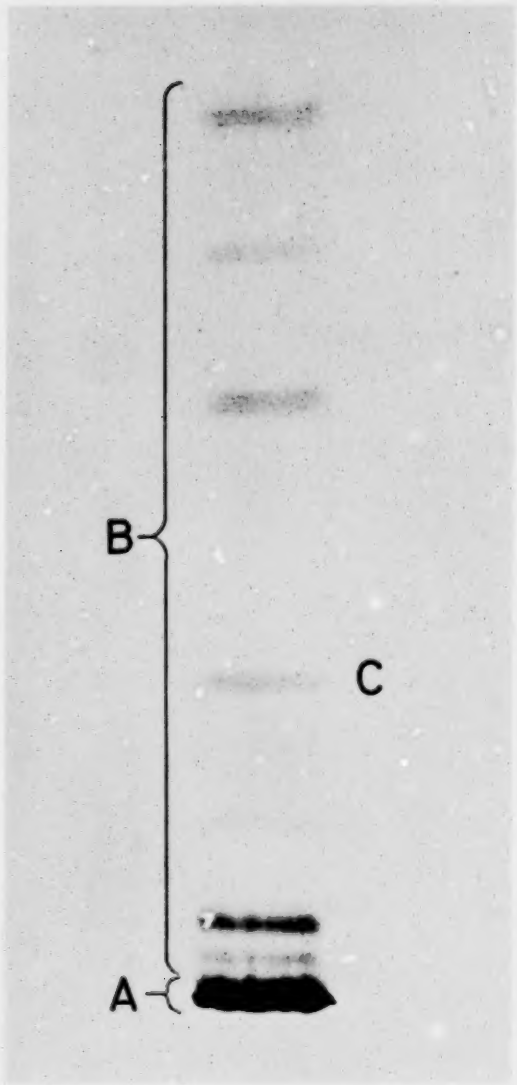

Figure 4 - Radioautogram of extract from Papaver bracteatum
(See Figure 3 C and Table 1).

A. Unconverted isotope remaining at the starting line.

B. Radioactive metabolites derived from the precursor.

C. Location of thebaine.

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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM

No. 23

Report of the Fourth Working Group on Papaver bracteatum 1/

Rennes, France

17 - 21 May 1976

1/ This meeting was held with the financial support of the United Nations Fund for Drug Abuse Control.

GE. 76-6477

REPORT OF THE FOURTH WORKING GROUP ON PAPAVER BRACTEATUM

1. During recent years, considerable interest has developed in Papaver bracteatum as a known source of thebaine and thus, by conversion, of codeine[2]. Though at the present time world codeine supply and demand are approximately in balance, reserve stocks in some countries are insufficient to compensate for one poor crop year. Meanwhile, medicinal needs for codeine are increasing year by year. Sources of codeine other than Papaver somniferum would therefore be desirable and the United Nations is interested in P. bracteatum as one such source.

2. A collaborative research project on this poppy was initiated by the United Nations Narcotics Laboratory and investigations are being carried out in many countries. Working Groups of experts were held in Geneva (1972)[3], Teheran (1973)[4] and Beltsville, Md., USA (1974)[5].

3. The fourth Working Group of experts[6] was convened in France from 17 to 21 May 1976 with the financial support of the United Nations Fund for Drug Abuse Control. Facilities for the meeting were provided at the Station d'Amélioration des Plantes near Rennes through the courtesy of the Director, Dr. J. Morice. The final discussions on 21 May 1976 were held at UNESCO Headquarters in Paris.

2/ Codeine is the most widely used anti-tussive and it is also extensively used as a mild analgesic. At present, codeine is mostly derived by the process of methylation from the morphine obtained from the opium poppy and about 90 per cent of legally produced morphine is converted into codeine.

3/ See report, document ST/SOA/SER.J/1.

4/ See report, document ST/SOA/SER.J/2.

5/ See report, document ST/SOA/SER.J/15.

6/ The list of participants and observers is given in Annex I.

4. The meeting was opened by Mr. M. Hives, Chief of the Plant Improvement Department of the French National Institute of Agronomic Research who welcomed the Group and stressed the importance of the research being carried out on P. bracteatum.

5. Dr. J. Morice was elected Chairman by acclamation. Dr. O.J. Braenden represented the United Nations and Miss E.S. Lumsden was the Executive Secretary of the Working Group.

6. It was noted that considerable progress had been made since the initiation of the research, particularly since the third Working Group.

7. Differences in growth habit, age at first flowering, and thebaine content were reported as these relate to genetic variations of P. bracteatum and to the wide range of ecological conditions under which the plant is being grown experimentally.

8. Consideration was given to methods of establishing stands whether by (a) direct seeding or seedling transplants or (b) vegetative reproduction by cuttings (longitudinal sections through root crown and upper root or transverse sections of root). The importance of high quality seed with good germination rate was recognized as an essential component of stand establishment. Consideration was also given to the effect of the depth of planting on seedling emergence 1/. Earlier experiments had shown that the best temperature range for germination of P. bracteatum seed was between 15 and 25°C 8/. This has since been confirmed at a number of locations. Maximum and minimum temperatures play an important role during the initial year of stand establishment. Temperatures of -10°C or below (without snow cover) and of +40°C or above killed unprotected plants. However, these young plants can be effectively protected from such temperature extremes by appropriate mulches or coverings.

1/ See document ST/SOA/SER.J/15, para 13.

8/ ibid, para 12.

9. The Group recognized the importance of evaluating plant spacing in terms of thebaine yield per unit area and consideration was given to spacing within and between rows. Some locations reported best results in terms of weed control and yield of capsules when plant density was high but it was recognized that optimum spacing may vary from location to location depending on such factors as available moisture, weed problems, methods of weed control and harvesting methods.

10. Broadcast seeding can be a simple approach to stand establishment but this may have disadvantages, particularly in connexion with weed control and the use of herbicides on the one hand and mechanical cultivation on the other. It was noted that, at the present time, there is no herbicide which adequately meets all requirements. However, reports indicated that Azulam [9] can be fairly effective when properly applied. Results of research on the use of the activated carbon method [10] of weed control were presented to the meeting. The report clearly demonstrated the potential of this method for establishing P. bracteatum seedlings from direct sowing. This method has been used successfully with other small seeded species.

11. There were several reports on the application of herbicides during the plant dormancy period. This can be done without detrimental effects on P. bracteatum. Paraquat [11] and diquat [12] had been used effectively during this period when there is no active growth taking place.

9/ Methyl sulfanilyl carbamate.

10/ The seeded rows are protected by a narrow band (about 5 cm) of activated carbon, then a general pre-emergence herbicide is applied over the entire surface of the field at the appropriate rate. The carbon deactivates the herbicide so that seedlings come up through the carbon undamaged.

11/ 1,1'-dimethyl-4,4'-dipyridinium dichloride (or methosulfate).

12/ 9,10-dihydro(8,10a)diazoniaphenanthrene dibromide.

12. It was generally agreed that P. bracteatum is a highly cross-pollinated species. The controlling mechanism appears to be self incompatibility either genetic or somatic, however, there is some evidence of a low level of self pollination.

13. The perennial nature of P. bracteatum was discussed and several reports confirmed that the productive life of this plant is at least eight years [13/]. In fact, there is experimental data indicating that yield of thebaine per plant increases with plant age.

14. Mention was made of the possibility of using seeds in paper strips to facilitate precision in plant spacing, and perhaps enhancing weed control and fertilizer application. Use of pelleted seed has met with mixed success.

15. Several participants reported on disease and insects attacking the various parts of the P. bracteatum plants. It was recognized that chemicals would control certain of these pests but the primary objective should be the development of disease and insect resistant varieties which would, at the same time, have high alkaloid yield.

16. The meeting was informed by several participants that the thebaine concentration in capsules varied with the stage of capsule maturity. It was generally agreed that the concentration reached a peak two to four weeks after flowering. Following this period of development, the percentage of thebaine decreased until just prior to capsule maturity when there was an additional accumulation of the alkaloid. It was also reported that a portion of the thebaine in the capsule is probably in a bound form in the post-flowering period. It was noted, however, that the bound form disappears and becomes part of the total thebaine prior to capsule maturity. Meconic acid was found to be present but probably not in the bound form.

13/ This was the longest period of controlled growth of any P. bracteatum stand.

17. Reports were presented on progress in selecting and breeding of P. bracteatum populations with high thebaine content and other desirable characteristics. Data were given showing the range of genetic variability within the species and the opportunities for plant improvement. The identification of plant selections from Arya II [14/] with high thebaine content was reported. In certain selections significant positive correlations were found between size of capsules and thebaine percentage. Experimental evidence was provided which indicates that thebaine content is a heritable characteristic. Also it was reported that the magnitude of variation in thebaine content of capsules was greater between plants than within plants.

18. It was noted that preliminary research was in progress in connexion with thebaine production through tissue culture. It was reported that, after the tenth generation, thebaine comprised 0.1% of the dry matter. These investigations include the study of various rhythms of culture in different types of environment, the analysis of mitotic activity and of the relationship within the cell between proliferation and biosynthesis. Studies were also being made on the production and selection of improved cell strains.

19. Preliminary data show that ^{14}C-labelled phenylalanine, tyrosine, glycine and urea when applied to capsule, leaf and stem surfaces of P. bracteatum

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14/ At the second Working Group on P. bracteatum considerable interest was expressed in new collections from the Mahabad region in the west of Iran. The capsules of these plants have the highest content of thebaine. The pigment and resin content is low and no other alkaloid was detected in the dry capsules. It was thus possible to obtain pure thebaine relatively easily. In view of the importance of this discovery, the second Working Group felt that it would be desirable to nomenclaturally distinguish collections of the Alborz Mountains from those of the Mahabad region by referring to the former as "Arya I" and to the latter as "Arya II". (see document ST/SOA/SER.J/2)

are absorbed and at least partially utilized in alkaloid biosynthesis. Radioactive thebaine was found primarily in the capsules of the plants treated with each of the four precursors. There was little radioactivity present in the extracts from the root stem transition zone or leaves. No radioactive compounds were found in the root. It was suggested that future experiments on biosynthesis of thebaine should include precursors with small molecules, in general not belonging to the primary metabolites.

20. The meeting was informed of the structure of articulated laticifer or latex cells in P. somniferum; similar structures were found in P. bracteatum. The latex cell contains numerous vesicles, organelles, ribosomes and a single nucleus. As these cells mature, thin areas develop in walls of contiguous cells. These thin areas become perforated so that the cells are interconnected and eventually, as more and more cells are connected, form an anastomozing system of latex vessels (articulated laticifers). They are derived from endoplasmic reticulum and usually develop a thickened area or cap prior to perforation of the latex cells. The vesicles represent the major component of exuded latex. It is not yet known whether the alkaloids are synthesized within the vesicle, on the vesicle membrane, within the cap or elsewhere in the latex cell.

21. The Group felt that suitable methods are now available for the determination of thebaine in P. bracteatum material. The reference samples prepared by the UN Laboratory had been analysed in various laboratories by the method recommended by the third Working Group [15] and the results obtained were in good agreement.

22. Several modifications of the method that had been recommended by the third Working Group were suggested [16]: freeze-drying of material had been found

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15/ See Annex II.

16/ These modifications will be described in a document to be issued in the ST/SOA/SER.J/.. series.

to give higher thebaine values than air-drying at 60°C and specification of the particle size of samples is necessary in order to obtain reproducible and comparable results. It was desirable to have uniform milling devices and procedures. The optimum temperature for extraction was 15°C or lower and continuous elution was of advantage. At the present time, gas-liquid chromatography seems the preferred technique but it was noted that promising results had been obtained with high pressure liquid chromatography.

23. The thebaine content of the dried ground capsules was relatively stable for a considerable time. However, if any oil was present from the seeds the amount of thebaine diminished fairly rapidly.

24. A pilot study had been carried out on 3.1 metric tons of Arya II capsules to determine the feasibility of commercial extraction of thebaine from P. bracteatum. No difficulties had been encountered with the process used.

25. Considerable interest was shown in the presentation of a review of methods for the conversion of thebaine into codeine and it was suggested that this review should be included in the report of the Group 17/. It was also noted that thebaine could be converted directly into hydrocodone and dihydrocodeine with high yield.

26. Information presented on the percentage of oil extracted from P. bracteatum seed by commercial type presses confirm the oil content of P. bracteatum seed reported to the third Working Group 18/. The oil and seed meal from a large scale (10 metric tons) pilot study are being evaluated for their food, feed and industrial values.

27. The Working Group was informed of a new alkaloid-producing perennial plant found at high elevations in Iran. It was reported to be similar to

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17/ See Annex III.

18/ See document ST/SOA/SER.J/15, Annex VI.

Papaver fugax and Papaver caucasicum in morphological characteristics. It was noted that a new name had been proposed but not published. Preliminary analyses show that all parts of the plant contain approximately 1% thebaine with small quantities of fugapavine and two or three other minor alkaloids. Thebaine constitutes 95% of the total alkaloid component and appears to be equally distributed throughout the plant. At the present time, there is no evidence that higher concentrations of alkaloid accumulate in the capsules as compared with other parts of the plant, as in P. bracteatum. The plant has a single stalk reaching 1½ to 2 metres in height on which is produced 50 to 100 small pink flowers. Although the capsules are very small, the brownish seeds are 3 to 4 times larger than those of P. bracteatum.

28. In summarizing its debate, the Group reviewed the results achieved since the inception of the project in December 1972. The meetings of the Working Group together with the rapid publication avenue provided by the United Nations Narcotics Laboratory had helped all concerned to focus resources on problem areas and to avoid unproductive lines of research and wasteful duplication of effort.

29. There had been significant achievements in several aspects of the research and development of P. bracteatum, particularly in connexion with:

- methods of cultivation;
- the determination of thebaine in P. bracteatum material;
- semi-industrial processing of seeds for oil and protein and extraction of thebaine from capsules.

30. However, further investigations are desirable in such fields as:

- control of weeds, pests and diseases;
- plant growth requirements (for example, nutrition, water, photo-period, thermo-periods, spacing);

- harvesting practices (with special reference to large scale production);
- pilot studies to determine the processing technologies needed for commercial production [19];
- breeding and selection;
- morphological and anatomical studies relating to biosynthesis, translocation and metabolism of thebaine (including precursor studies for yield improvement).

31. The Group agreed that it would be highly desirable and extremely useful if the United Nations Narcotics Laboratory were to continue its leadership role in coordinating the research on P. bracteatum and that it should determine how this coordination can be most effectively carried out in the future.

32. Appreciation was expressed on the part of the observers for the opportunity to participate in the meetings of the Working Group [20]. The Group also recorded its appreciation of the visits that had been arranged to experimental cultivations of P. bracteatum.

33. Dr. C. Vaille, Inspector General of Social Affairs and Representative of France on the Commission on Narcotic Drugs and Mr. T. Francfort, Chief of the Narcotics Bureau of the French Ministry of Public Health, participated in the closing session of the meeting. Dr. Vaille underlined the concern at the international level in ensuring adequate supplies of codeine for medical purposes while, at the same time, avoiding over-production. The Commission on Narcotic Drugs was therefore especially interested in the research on P. bracteatum. In commending the results being achieved Dr. Vaille credited much of the progress to the close international collaboration as exemplified by this and previous meetings of the Working Group.

19/ A preliminary pilot study has indicated that this technology may be of an order of complexity similar to that for poppy straw but further data are necessary.

20/ See Annex IV.

ANNEX I

FOURTH WORKING GROUP ON PAPAVER BRACTEATUM

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+ + + + + + +

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Mrs. I. Bouthiaux (Administrative Assistant to the Group)

ANNEX II

At the third Working Group on Papaver bracteatum it was recommended that the following method be used for the determination of thebaine */:

1. Drying of Plant Tissue:

It was recommended that plant tissue be oven dried at 60°C to a constant weight as soon as possible following collection. Several members took exception to this procedure noting that thebaine may be lost using this protocol and that freeze-drying of samples would possibly be better. However, not all members had access to freeze-drying equipment. It was suggested that tissue be cut-up to facilitate oven drying, especially root tissue.

2. Particle Size:

It was recommended that samples be powdered so that all the sample passes through a 0.5 mm mesh and is thoroughly mixed immediately before weighing for analysis. Weight correction due to hygroscopic water absorption should be made by heating a small portion to 105°C and re-weighing at the time of analysis.

3. Sample Size for Analysis:

It was recommended that 0.1 to 0.5 grams of sample be used in analyses.

4. Extraction Solvent:

Acetic acid (5%) was suggested as the extraction solvent but would be considered tentative until fully evaluated by the members.

5. Procedure:

(a) Samples should be agitated for 30 minutes in 25 ml of acetic acid (5%) solution. It was recommended that shorter or longer agitating times be evaluated for efficacy of extraction technique.

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*/ See document ST/SOA/SER.J/15, Annex V.

(b) Filter eluent through a sintered glass funnel and repeat extraction two further times. Combine the filtrates.

(c) Add 25 ml of $CHCl_3$ and adjust the pH of the aqueous phase to pH 9.5-10 with concentrated NH_4OH with agitation. Separate $CHCl_3$ phases.

(d) Repeat extraction of the aqueous phase with one 25 ml portion of $CHCl_3$ followed by two 15 ml portions of the same solvent.

(e) Combine the $CHCl_3$ phases and evaporate to dryness preferably in vacuo at 40°C.

(f) To the flask containing the residue, add a known volume of ethanol containing an appropriate quantity of internal standard. Suggested internal standards include tetrahydrothebaine, cholesterol acetate, antipyrine and laudanosine. Use of one or more internal standards may be appropriate for certain analytical applications.

6. Gas-liquid Chromatography:

It was recommended that deactivated glass columns or glass-lined steel columns be used to avoid decomposition of thebaine (via metal catalysis). It was suggested that the columns be packed with 100/120 mesh Gas Chrom Q coated with 2-3% OV-17 liquid phase or their equivalents. Suggested operating temperatures should range between 260-280°C with either nitrogen or helium as carrier gas.

7. Results:

Results should be calculated on the basis of moisture-free content of plant tissue after drying at 105°C */.

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*/ Thebaine standardization: check the purity of the standard thebaine by determining the $E_{1\ \text{percent, 1 cm}}$ in 0.1N HCl at 285 nm or at the λ max.

ANNEX III

CONVERSION OF THEBAINE INTO CODEINE

by François Krausz

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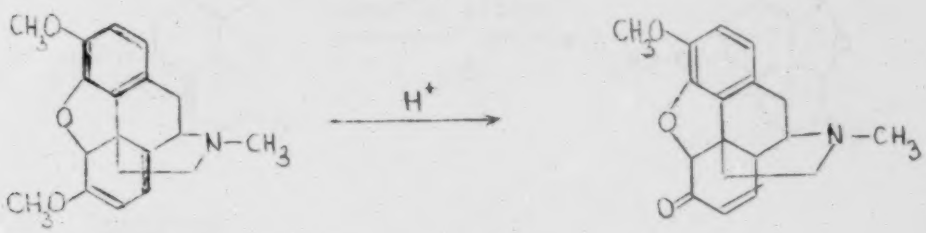
The need for converting thebaine into codeine first became apparent in the search for ways of making profitable use of thebaine, a by-product which is obtained in considerable quantities during the separation of the alkaloids of opium. The fairly recent discovery of a thebaine poppy, Papaver bracteatum, further emphasized the need for such a process. From the outset, it was logical to assume that the process would involve codeinone as an intermediate.

At the beginning of this century, Knorr and Hörlein observed that thebaine, when treated with dilute sulphuric acid, produces a highly complex mixture, from which it is possible to isolate codeinone, with a very low yield (figure 1).

FIGURE 1



THEBAINE CODEINONE

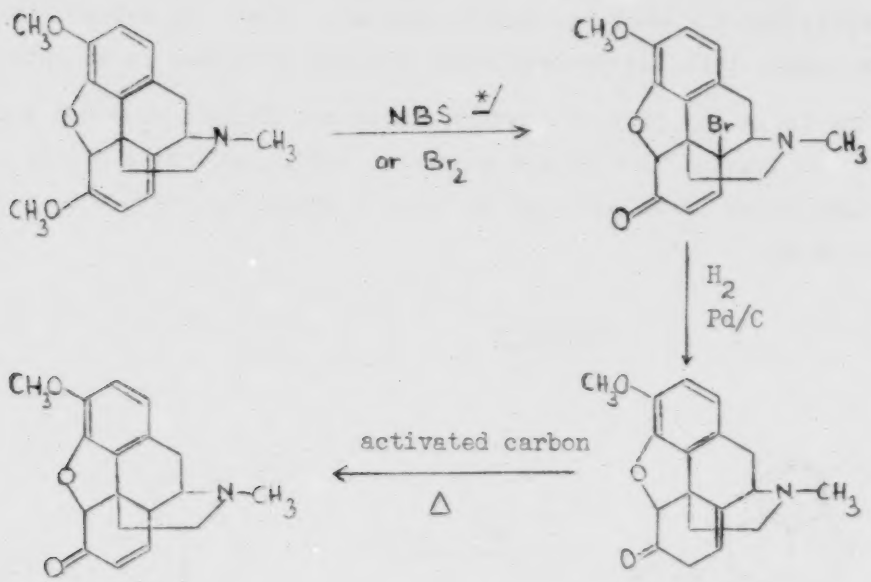
Knorr, Hörlein, Ber. (1906), 39, 1409
Schöpf, Hirsch, Ann. (1931), 489, 224

This discovery was of great theoretical importance since it shed some light on the relationships between the alkaloids of opium; but its practical value was nil because yields were low and isolation was difficult.

Also, the first practical method of reducing codeinone to codeine was not elaborated until the 1950s.

Great progress in the conversion of thebaine into codeinone was achieved in the work of Conroy (figure 2)

FIGURE 2



NEOPINONE

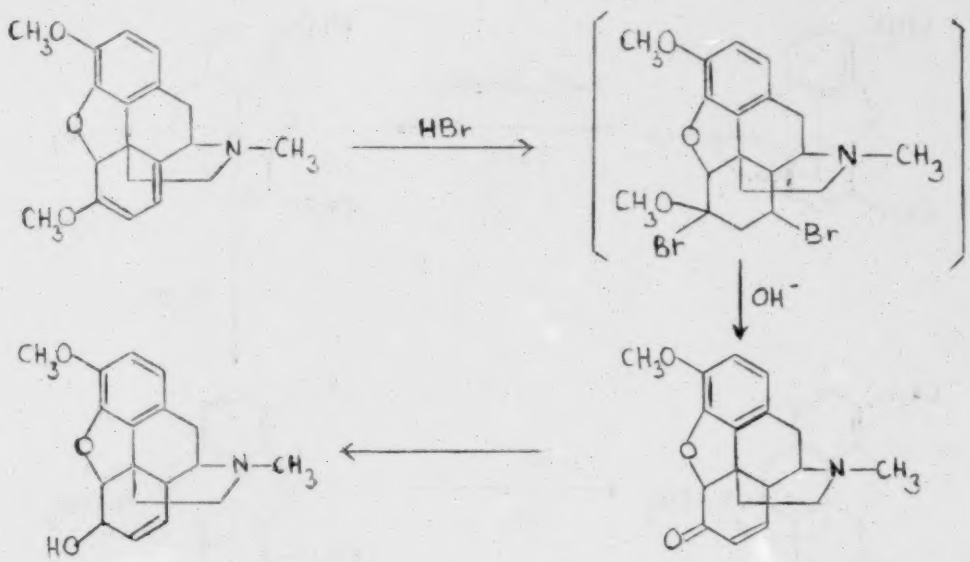
Conroy, J. Amer. Chem. Soc. (1955), 77, 5960
Krausz, Rüll. Bull. Soc. Chim. (1960), 2148

*/ N-bromosuccinimide

who discovered a clean method of converting thebaine into 14-bromocodeinone; this substance, when subjected to catalytic hydrogenation, was converted into neopinone, which itself isomerizes under various conditions into the conjugated ketone, codeinone. The total yield is about 37 per cent. However, the extension of this method to yield quantities larger than those produced under laboratory conditions would seem rather disappointing.

Our reflexions on the development of the chemistry of thebaine led us, at the beginning of the 1960s, to elaborate an extremely simple method of converting thebaine into codeinone (figure 3).

FIGURE 3



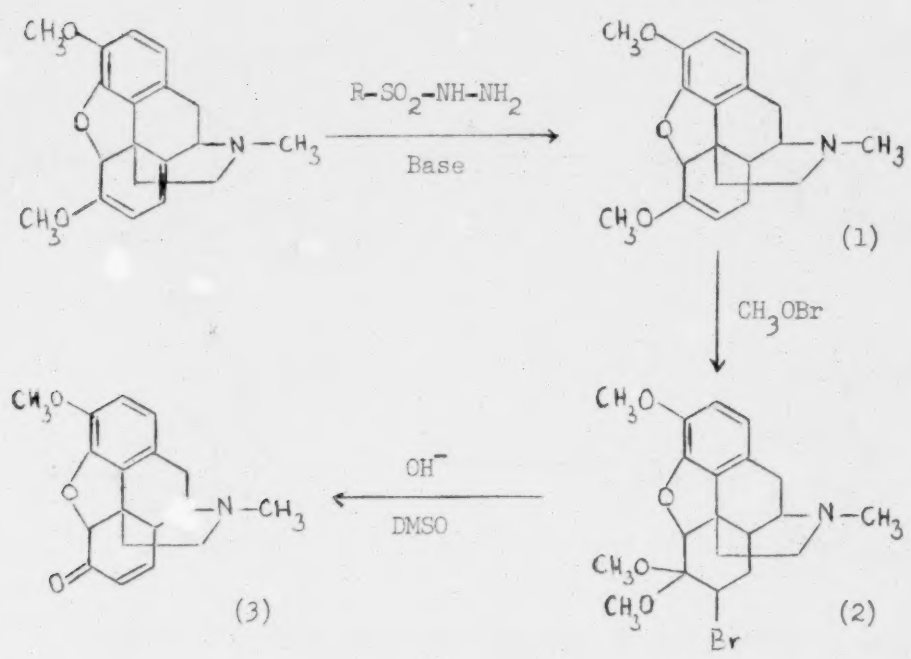
CODEINONE: SEPA (inv. F. Krausz), Fr. pat. 1.290 174 (1961)
Gavard, Krausz, Rüll, Bull. Soc. Chim. (1965) 486

CODEINE: Merck (inv. M. Gates), Fr. pat. 1.103 238 (1955)
Krausz, Gavard, C.R. Acad. Sc. (1962) 254, 3101

When treated with hydrobromic acid in an anhydrous medium, thebaine, after hydrolysis, produces codeinone in a single step (the intermediate product, which is shown in brackets is not isolated) and with very satisfactory yields and purity. After the codeinone has been reduced to codeine, the overall yield from the conversion of thebaine into codeine is of the order of 70 per cent.

Very recently, we noted an ingenious method of converting thebaine into codeine, which is illustrated in figure 4.

FIGURE 4



(1) Eppenberger, Warren, Rapoport, Helv. Chim. Acta (1968) 51, 393
Macfarlan Smith Ltd., Fr. pat. 2.117 924 (1972)
(2) Rapoport, Lovell, Reist, Warren (1967), 89, 1942
(3) Macfarlan Smith Ltd., Belg. pat. 827 568 (1975)

The use of aryl sulfonyl hydrazide makes it possible to produce the diimine HN = NH, which was first used by Rapoport et al., in the reaction medium reasonably safely. The thebaine is reduced to dihydrothebaine, with excellent yields. The latter, upon reaction with CH_3OBr, is converted into 7-bromoacetal dihydrocodeinone (ref.2). From this bromoacetal using sodium or potassium in a DMSO medium, it is possible to obtain codeinone acetal, from which codeinone is obtained by acid hydrolysis.

This ingenious series of reactions produces interesting yields. However, in view of the relative complexity of the operations and the cost of some of the raw materials, we believe that conversion by the process described earlier would be more economical.

ANNEX IV

Statement made by Mr. Clealand Baker on behalf of representatives of manufacturing companies present as observers

As a representative of a pharmaceutical manufacturer that uses a large quantity of codeine in formulating medicinal products, I have appreciated the privilege of attending the last two U.N. Papaver bracteatum meetings as an observer. I have been impressed with the fine cooperation of the Working Group members, and the effective coordination of the studies by the U.N. Laboratory under Dr. Braenden's direction.

The report given at this meeting covering the results of the chemical analysis of the reference samples of P. bracteatum capsules for thebaine content, is a good example of the productive value of these coordinated studies. I know of no similar scientific development project that has moved along this rapidly, which may emphasize the importance of the effective U.N. Laboratory coordination of this international study group involving scientists from government, academia, and industry. I hope this fine cooperation will continue in future studies that are done on this plant.

P. bracteatum has the potential to contribute substantially to the world community in several ways - to improve the raw material supply for codeine, to stabilize or possibly reduce the rapidly rising price of codeine, and by providing a raw material source that is free of naturally occurring morphine, to be useful in reducing the drug abuse potential from the alkaloid source.

I wish to congratulate the U.N. Laboratory and the Working Group on their achievements, and also Dr. Morice, as President of the Conference, for his fine conduct of the meetings. My appreciation also to Mr. Dumont and Mr. Candillon for their hospitality during the Conference.

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SCIENTIFIC RESEARCH ON PAPAVER SPECIES AS SOURCES OF CODEINE, MORPHINE AND THEBAINE 1/

No. 24

Research in Norway on Papaver species designed to improve the yield of phenanthrene alkaloids and to produce quality seeds

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1/ This ST/SOA/SER.J/.. series of documents was previously entitled "Scientific research on Papaver bracteatum".

General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

GE. 77-1372

INTRODUCTION

The following species and varieties of Papaver were used in the research programme: Papaver bracteatum (Arya I); Papaver bracteatum (Arya II); Papaver somniferum (12 varieties); Papaver orientale and Papaver pseudo-orientale.

Test cultivation of Papaver bracteatum (Arya I) was started in 1968. Plant improvement experiments (γ-radiation of seeds before planting and polyploidation experiments) were carried out over the last 3-4 years. Precursor incorporation experiments, with labelled and non-labelled precursors, were started in 1975 and went on in 1976. Analyses of seeds from Papaver bracteatum (Arya I), and analyses of the alkaloid content of several samples of the test cultivated species, have been carried out, or are in progress. Investigations on the low-molecular carbohydrate fraction, the higher carbon sugars and the water-soluble polysaccharides of Papaver somniferum have been performed and published. Comparative investigations on the organic acids in the above mentioned species of Papaver are in progress, and studies on the influence of ecological factors on the alkaloid content of several Papaver species was started last summer.

Appendix I shows maps of the experimental plots during the summer 1976.

Reports on work and results obtained prior to the 1976 season were given at the Bracteatum symposia in Geneva, December 1972; in Teheran, September 1973; in Beltsville, October 1974 and in Rennes and Paris, May 1976. Appendix II includes a list of publications on opium and Papaver species by A. Nordal and coworkers.

EXPERIMENTAL RESULTS

In the following a very brief review is presented on the research by our group on Papaver bracteatum and Papaver somniferum, to improve the yield of codeine, morphine and thebaine.

As is known, investigations of this kind involve months of field work, followed by harvesting and preservation of hundreds of samples, before the analyses can be completed and the value of the experiments determined.

We are now at the stage where chemical and radio-chemical analyses of our samples from the 1976 harvests are in progress, and I estimate that it will take at least two months to complete the analytical part of the programme.

1. Papaver bracteatum (Arya I)

As mentioned above test cultivation of this species was started in 1968. The main problem at that time was to find out if the plant could be cultivated in Norway. We know today this is quite possible if certain precautions are taken to insure that the roots of the plants survive during the winter season.

γ-Radiation experiments

The plants used for this investigation were raised from seed type UNB-2, 1972. (The same type of seeds was used also for the polyploidation experiments).

The radiated seeds were supplied by Dr. O.J. Braenden, U.N. Narcotics Laboratory, Geneva, in 1972. We used four samples that had been given 20, 40, 60 and 80 Rad respectively. Seeds germinated from the two lower levels of radiated samples only.

About 70% of the plants which grew died following transplanting or during the first and second winter in the field. The radiated seeds gave plants which, as a rule, were less vigorous than plants from non-radiated seeds (control plants) and only a few of them gave flowers and fruits in 1974, the second year after planting. During the winter 1975-76, there was much frost without snow cover, in the Oslo area and about one half of the remaining plants died. Of the plants which survived (about 30 all together) approximately 50% flowered and gave ripe fruits in 1976. They were collected and will be analysed for their thebaine content and for certain other constituents to determine to what degree they differ from the control plants.

Polyploidation experiments

Seeds from P. bracteatum (UNB-2, 1972) were treated with varying concentrations of colchicine before and during germination to obtain plants with a higher chromosome number than the parent plants. The experiments which were started in 1972 are still underway. Analyses have shown that some polyploidation has taken place, but the analytical part is not yet completed.

Chemical composition of the seeds

Table I shows comparative analyses of the content of lipids, proteins and carbohydrates in the original seed type UNB-2, 1972 and in the corresponding seeds derived from plants raised in Norway.

Table I

Type of seeds	Fat (ether extract) %	Proteins %	Carbohydrates %
Seeds of Papaver bracteatum UNB-2, 1972 from Iran	27,0	27,3	40,4
Seeds of Papaver bracteatum UNB-2, 1972 raised in Norway	18,4	26,0	49,9

The rather low content of fat and the rather high content of carbohydrates in the seeds raised in Norway, indicate that the Norwegian seeds were not quite ripe when analysed, but germination tests have shown that fertile seeds of P. bracteatum can be produced in Norway.

2. Papaver bracteatum, UNB-17 (Arya II)

The first seed sample of this selection was received from the U.N. Narcotics Laboratory in December 1973. The plants in Plot C (see Appendix I) were raised from these seeds.

In 1975 plants from Plot C were used for precursor incorporation experiments where labelled compounds of L-phenylalanine, L-tyrosine, L-glycine and urea were applied as precursors. A preliminary report on this investigation is given in UN Secretariat publication ST/SOA/SER.J/22, 30 April 1976. A paper on the same subject has been accepted for publication in Acta pharmaceutica Suecica.

Analyses carried out on capsules from Plot C (Arya II), 1975 crop, showed a thebaine content of 4.0%. Corresponding analyses of capsules from Plot A (Arya I, 1975) showed a thebaine content of 1.34%.

The successful experiments with labelled precursors in 1975 were followed, in 1976, with non-labelled precursors in order to find out if the expected raise in the relevant alkaloids content would be of such a magnitude that it could be determined by means of conventional chemical methods.

In 1976, Plot C (Arya II) was divided into four equal sections and the plants were sprayed once a week from June 3 to August 2, 1976 with the precursor solutions shown in Figure 1. On August 9 the plants were harvested and air dried. Analyses of the plant material are in progress.

3. Papaver somniferum

1. Plot B (see Appendix I) was sown in 1976 for two reasons:

(a) to produce seeds of Norwegian origin for ecological investigations;

(b) to perform precursor incorporation experiments with labelled and non-labelled precursors with this species in the same way as with Papaver bracteatum (Arya II).

The seeds used for this plot were of Turkish origin, and a good yield of fertile seeds (about 1 kg) was obtained.

Precursor incorporation experiments with labelled compounds (urea, phenylalanine and glycerine) and with a non-labelled compound (urea) were performed in the same way as described for Arya II, and the analyses of the plant material are in progress.

2. Plot H and Plot I (see Appendix I)

In both plots the same 12 varieties of Papaver somniferum were grown. Plot H was located in the Oslo area and Plot I north of the Arctic circle.

The reason why these plots were started in 1976 was to perform some pilot experiments in connexion with the above mentioned ecological investigations. Plot H was also expected to give more varieties of seeds for the same investigations.

The plants from the two plots were harvested at the end of September 1976 and dried. The seeds from the different varieties will be taken care of, and the aerial parts of the plants will be subjected to comparative analyses.

RESULTS AND DISCUSSION

During the last five years a good deal of work has been carried out in Norway on Papaver bracteatum and other Papaver species. The main scope of this work has been, in close collaboration with United Nations Narcotics Laboratory, to perform scientific investigations to improve the yield of phenanthrene alkaloids in certain poppy species.

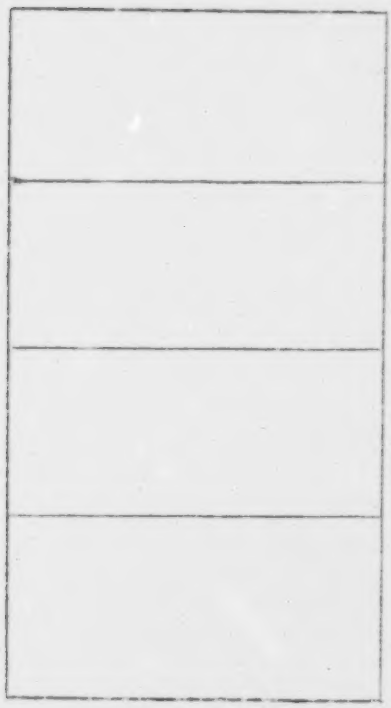
The investigations required a considerable amount of living plants and fresh and preserved material, which gradually was provided by cultivation of the relevant species at different places in Norway.

This first important, but time consuming, phase of the programme is now over, and the results are starting to accrue. For the time being two scientists from our staff, J.K. Wold, Ph.D., and B. Smestad Paulsen, Ph.D., plus a technician and myself are involved in the project.

The most promising results so far were the precursor incorporation experiments mentioned above. Also our chemical research might be important in elucidating the metabolic pathways, which in live plants lead to the production of the useful alkaloids (see the reference list in Appendix II).

Figure 1

Plot C (Arya II) 1976



Section 1: Control plants
(urea 1% in water)

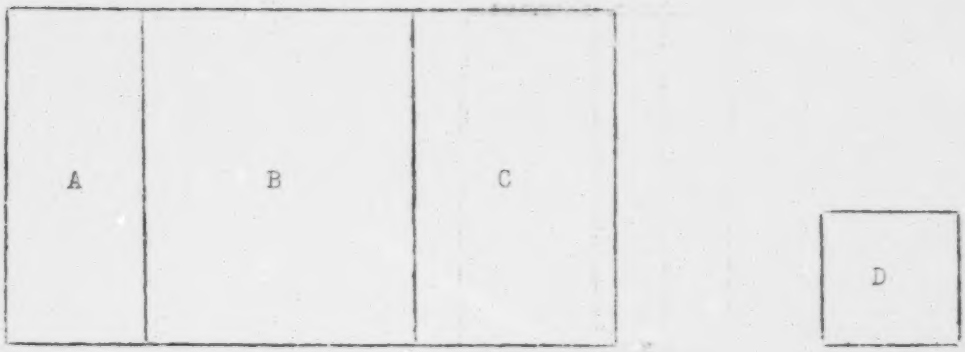
Section 2: Urea 1% in 1.0%
dimethylsulfoxide (DMSO)

Section 3: Urine : H_2O (1:1) in 0.1%
MON 0011

Section 4: Urea 1% in 0.1%
MON 0011

APPENDIX I

Research plots for Papaver species at Botanical Garden,
Tøyen, Oslo, Norway 1976



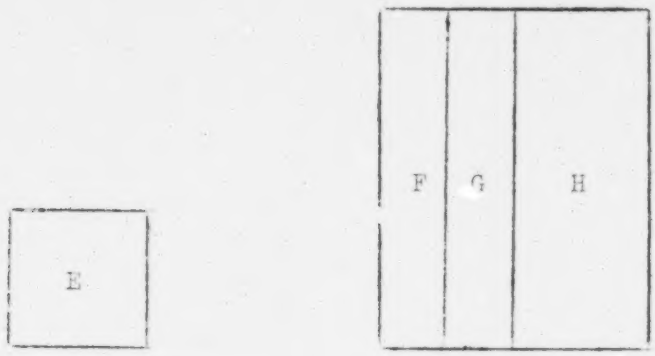
Plot A (100 m^2) : Papaver bracteatum (Arya I). Started 1968 for test cultivation, combined with chemical analyses of seeds, alkaloid content and organic acids.

Plot B (200 m^2) : Papaver somniferum. Started 1976. Used for labelled and non-labelled precursor incorporation experiments, for chemical analyses of alkaloids, organic acids and carbohydrates, and for seed production (see Plot H).

Plot C (150 m^2) : Papaver bracteatum (Arya II). Started 1974. Used for labelled and non-labelled precursor incorporation experiments and for chemical investigations.

Plot D (15 m^2) : Papaver bracteatum (Arya I). Started 1971 with γ -radiated seeds.

Research plots for Papaver species at the Phytotron area,
Blindern, Oslo, Norway 1976



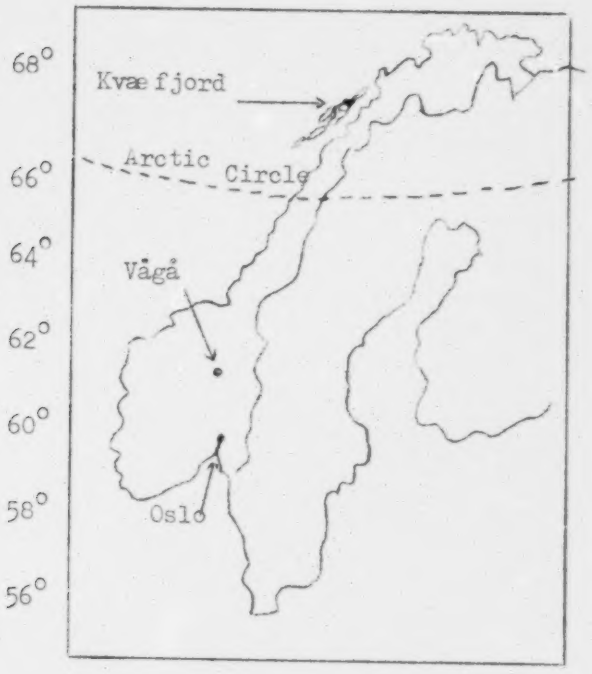
Plot E (15 m^2) : Papaver bracteatum (Arya I). Started 1972 for polyploidation experiments.

Plot F (50 m^2) : Papaver orientale. Started 1973, and used for comparative purpose (to study similarities and differences in the alkaloid and organi acid content of this species and the closely related Papaver bracteatum).

Plot G (50 m^2) : Papaver pseudo-orientale. Started 1973 and used in the same way as Plot F.

Plot H (100 m^2) : Papaver somniferum (12 different varieties). Started 1976. Intended for comparative analyses of alkaloid content (see Plot I, Kvæfjord), and for supply of seeds for ecological investigations.

Research plot for Papaver species at Kvaefjord, north of the Arctic Circle



Map of Norway, showing the location of Plot I, at Kvæfjord, north of the Arctic Circle.

Plot I (100 m^2) : Papaver somniferum (the same 12 varieties which are mentioned under Plot H).
Small "pilot" experiment to find out if it might be worthwhile to include this locality in the planned ecological programme of 1977.
(see Plot H).

APPENDIX II

List of publications and reports on opium and Papaver species by Arnold Nordal and coworkers

1. Nordal, A.: Natural and synthetic drugs with morphine-like effects evaluated from a pharmacognostic point of view. Bull. on Narcotics, VIII, No.1 (1956).

2. Nordal, A.: Final report covering study and possible exploitation of pharmaceutical raw materials available in Burma. Prepared for the Government of the Union of Burma, Rangoon (1959).

3. Nordal, A., E. Ottestad, E. Brochmannn-Hanssen and Dagrun Øiseth: The water-soluble carbohydrates of Papaver somniferum L. J. Pharmacy and Pharmacology, 11, 689 (1959).

4. Nordal, A. and M. Blyberg Falck: The assay, characteristics, composition and origin of opium. United Nations document ST/SOA/SER.K/131, 4 October 1969.

5. Nordal, A.: The medicinal plants and crude drugs of Burma. Medd. Norsk Farm. Selsk. 25, 127 (1963).

6. Nordal, A. and M. Blyberg Falck: Opium with 25 per cent morphine content produced in Norway. Norges Apot. for. Tidsskr., 72, 173 (1964).

7. Wold, J.K., B. Smestad, R. Winsnes and D. Resser: The water-soluble polysaccharide of opium poppy (Papaver somniferum L.). Acta Chem. Scand., 24, 1262 (1970).

8. Wold, J.K. and B. Smestad: The water-soluble polysaccharide of opium poppy (Papaver somniferum L.). Identification of acidic oligosaccharides from a partial acid hydrolysate. Acta Chem. Scand., 24, 2472 (1970).

9. Haustveit, G. and J.K. Wold: Higher-carbon sugars of opium poppy (Papaver somniferum L.). Acta Chem. Scand., 24, 3059 (1970).

10. Haustveit, G. and J.K. Wold: The low-molecular carbohydrate fraction of opium poppy (Papaver somniferum L.). Medd. Norsk Farm. Selsk., 31, 157 (1970).

11. Nordal, A.: Papaver bracteatum Lindl. Report on test cultivation and (plant) improvement experiments carried out in Norway during the years 1968-1972. First Working Group on Papaver bracteatum, Geneva, 12 December 1972.

12. Nordal, A.: A corresponding report at the Second Working Group on Papaver bracteatum, Teheran, 3 September 1973.

13. Nordal, A.: A corresponding report at the Third Working Group on Papaver bracteatum, Beltsville, Maryland, U.S.A., 7 October 1974.

14. Nordal, A.: Papaver bracteatum Lindl. Report on test cultivation, plant improvement experiments and feeding with alkaloid precursors. Fourth Working Group on Papaver bracteatum, Rennes and Paris, 16-23 May 1976.

15. Nordal, A. and J. Kvalevåg: En undersøkelse av alkaloidene i de norske fjellvalmue-artene Papaver lapponicum (A. Tolm) Nordh., Papaver laestadianum Nordh., Papaver dahlianum Nordh. og Papaver dubium L. Hovedfagsoppgave utført ved Universitetets Farmasøytiske institutt, Avd. for farmakognosi. Oslo, May 1974.

16. Nordal, A., B. Smestad Paulsen and J.K. Wold: Scientific Research on Papaver bracteatum. Precursor incorporation experiments in Papaver alkaloid biosynthesis. I. United Nations document ST/SOA/SER.J/22, April 1976.

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SCIENTIFIC RESEARCH ON PAPAVER SPECIES AS SOURCES OF CODEINE, MORPHINE AND THEBAINE 1/

No. 25

ONTOGENETICAL AND CYTOCHEMICAL STUDIES OF ALKALOIDAL VESICLES IN LATICIFERS OF PAPAVER SOMNIFERUM L. 2/

by: Craig L. Nessler and Paul G. Mahlberg

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Indiana University,
Bloomington, Indiana, U.S.A.

1/ This ST/SOA/SER.J/.. series of documents was previously entitled "Scientific research on Papaver bracteatum".

2/ This study represents a portion of a Ph.D. thesis in the Department of Plant Sciences, Indiana University, Bloomington, Indiana, 47401, U.S.A.
A similar manuscript was submitted for publication to the American Journal of Botany, September 1976. RNDD Registration Number PM0084537.

General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

INTRODUCTION

Laticifers in Papaver somniferum L. can be distinguished from other cell types at the ultrastructural level by the large irregularly shaped vesicles that fill the mature cell. Origin of these vesicles by the distention of endoplasmic reticulum was first suggested by Sárkány et al. (1964) and later by Thureson-Klein (1970), although vesicle ontogeny was not studied in detail. The occurrence of alkaloids in latex from poppy has been known from the classical literature and recently Fairbairn et al.(1974) reported that the alkaloids were associated with the vesicles within the vacuolar plasm of this cell. Dickenson and Fairbairn (1975) investigated the ultrastructure of these "alkaloidal vesicles" in both ultracentrifuged latex and intact plants.

In the present investigation the ontogeny of alkaloidal vesicles in laticifers of P. somniferum is described and cytochemical evidence presented for their origin from endoplasmic reticulum. Information derived from our current research program will contribute to a more complete understanding of the laticifer and its latex, and will contribute to our understanding of the mechanism of alkaloid formation whereby we may be able to increase the yield of alkaloids in this plant.

MATERIALS AND METHODS

Plants of Papaver somniferum L. cv. "Neuga" were grown from seed in the greenhouse. Seeds were also germinated on cheese cloth covered paper cylinders in glass jars to obtain straight radicles for study. Various portions of plant axes were fixed 8 - 12 hr at room temperature in one of the following fixatives: 1) 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2; 2) 1% glutaraldehyde, 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (Karnovsky, 1965); or 3) 4% glutaraldehyde, 0.7% picric acid in 0.1 M sodium cacodylate buffer, pH 7.0 (Turner, 1970). After several rinses with 0.05 M sodium cacodylate, pH 7.0, tissues were post-fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate, pH 7.0, for 2 hr, rinsed with glass-distilled water and prestained in 1% aqueous uranyl acetate at 4 C for 6 - 12 hr. Tissues were

then rinsed with water, dehydrated through an ethanol-acetone series and embedded in Spurr (1969) resin. Some tissues were fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2 for 2 hr and dehydrated and embedded as described above.

Procedures described above were modified for cytochemical electron microscopic investigations as follows:

1) Zinc iodide-osmium tetroxide (ZIO) impregnation. Tissues were fixed in the dark for 24 hr in a zinc iodide-osmium tetroxide solution (Niebauer et al. 1969). Tissues were rinsed with water, prestained with uranyl acetate, dehydrated and embedded as described above. A modification of this procedure consisted of treating tissues with Turner (1970) fixative for 8 - 16 hr before ZIO impregnation.

2) Osmium tetroxide impregnation. Tissues were fixed at room temperature in 1% osmium tetroxide in 0.05 M phosphate buffer, pH 7.2, for 1½ hr. After initial fixation, tissues were impregnated for 2 - 6 days with osmium tetroxide by placing them in foil covered vials containing unbuffered 2% osmium tetroxide at 40 C (Friend, 1969). Osmium tetroxide solutions were changed every 24 hr. Tissues were rinsed with water and prestained with unranyl acetate for 1½ hr at room temperature, dehydrated and embedded as described above. Thin sections were cut on a Porter-Blum MT-2 ultramicrotome, collected on 300 mesh copper grids, stained with lead citrate and unranyl acetate and examined with a Hitachi HU-11C electron microscope at 50 KV.

RESULTS

In early stages of differentiation, rough endoplasmic reticulum was abundant throughout the densely packed cytoplasm of laticifer initials. During laticifer differentiation this endoplasmic reticulum accumulated along the periphery of the plasma membrane in long folded sheets oriented parallel to the longitudinal wall (Fig. 1). Segments of this endoplasmic reticulum also occurred within a lobe of the nucleus (Fig. 4) and in smaller sheets surrounded by developing vesicles (Fig. 5).

Endoplasmic reticulum of cells in early stages of vesiculation were represented by rough endoplasmic reticulum alternating with smooth areas that lacked ribosomes (Fig. 2). Stacked endoplasmic reticulum was surrounded by high concentrations of

polysomes that appeared to be derived from the surface of rough endoplasmic reticulum as it was transformed to the smooth form (Fig. 1, 2).

Vesicles, which comprised most of the mature laticifer protoplast, were bounded by unit membrane (Fig. 5) and appeared to be derived from endoplasmic reticulum. Vesicles were derived from dilation of both smooth (Fig. 6) and rough (Fig. 7) endoplasmic reticulum, the latter losing ribosomes from its surface during dilation. Derivation of vesicles from dilation of endoplasmic reticulum is illustrated in Fig. 23.

Impregnation with the zinc iodide-osmium tetroxide (ZIO) reagent resulted in differential staining of laticifer vesicles as well as the vacuolar membrane of neighbouring cells (Fig. 8, 9). This staining resulted in selective deposition of granular electron-dense materials along the inner surface of these membranes. When ZIO impregnation was preceded by fixation with glutaraldehyde in picric acid, the ultrastructure of all cells was well preserved (Fig. 10). With this treatment, laticifer vesicles were selectively stained along their inner membrane (Fig. 11); adjacent cells showed occasional staining only within the perinuclear space and mitochondrial matrices (Fig. 12).

Laticifer vesicles (Fig. 13, 14) and endoplasmic reticulum (Fig. 14, 15) were selectively stained by osmium tetroxide impregnation. The forming face of dictyosomes and portions of endoplasmic reticulum in adjacent cells were also differentially stained (Fig. 16). As with the ZIO technique, only the inner membrane surface of vesicles was stained (Fig. 17).

As vesicles began to proliferate in the laticifer cytoplasm, black electron-dense regions or "caps" developed along their periphery (Fig. 19) by the condensation of small particles along the inner surface of the vesicle membrane (Fig. 1). These particles, which formed within the developing vesicles, appeared as a mixture of small electron-dense and electron transparent granular deposits at high magnification (Fig. 18). The relationship between these different particle types is not known at present.

Condensation of particles into caps occurred as vesicles enlarged (Fig. 1); however, the caps were formed before the laticifer was fully differentiated (Fig.19).

When tissue at the late vesiculation stage was fixed in buffered osmium tetroxide, the vesicle caps appeared enlarged and granular (Fig. 20, 21). Vesicle caps were not as conspicuous in mature laticifers as in earlier stages of development of this cell (Fig. 3, 22). The large size and close packing of vesicles in the mature laticifer protoplast made the caps difficult to recognize.

The origin and development of vesicle caps during the vesiculation process in the developing laticifer is illustrated in Fig. 23.

DISCUSSION

Results of our cytochemical investigation support the interpretation that all vesicles of the mature laticifer protoplast were derived from endoplasmic reticulum. These studies confirm the observations of Thureson-Klein (1970) who reported that vesiculation resulted from localized dilation of endoplasmic reticulum.

All the vesicles of mature laticifers in opium poppy are stained by ZIO impregnation as is the vacuolar membrane of neighbouring cells. The stain appears as electron-dense deposits along the inner surface of these membranes and is similar to the description reported by Marty (1973a, b) for the vacuolar membrane and provacuoles of Euphorbia characias cells.

Results of ZIO impregnation indicate that laticifer vesicles in poppy may be analogous to the central vacuole of other cells as suggested by Thureson-Klein (1970), since both membranes show a similar staining pattern with this procedure. However, this procedure does not conclusively demonstrate that these membranes originate directly from dilation of endoplasmic reticulum in view of the similar staining of dictyosome-derived vesicles reported by Dauwalder and Whaley (1973) and Marty (1973a, b).

In plants, fixation with glutaraldehyde followed by ZIO impregnation has been reported to stain the endoplasmic reticulum, proximal face of dictyosomes,

nuclear envelope, mitochondrial matrices and occasional plastid matrices (Dauwalder and Whaley, 1973; Susalla and Mahlberg, 1975). Application of this technique to opium poppy tissue resulted in the staining of all laticifer vesicles and occasional staining of the perinuclear space and mitochondrial matrices of adjacent cells.

Differential staining of laticifer vesicles and the nuclear envelopes of adjacent cells would seem to directly implicate the endoplasmic reticulum in vesicle formation, but such staining has also been reported for dictyosome vesicles in cortical parenchyma of Zea mays (Dauwalder and Whaley, 1973).

In other studies on plant cells, the ZIO technique has been found to stain the endoplasmic reticulum, nuclear envelope, and tonoplast of some impregnated cells (Dauwalder and Whaley, 1973; Marty, 1973a, b). The internal portions of lamellar membranes of chloroplasts are also differentially stained by ZIO (Dauwalder and Whaley, 1973; Susalla and Mahlberg, 1975) as well as the photosynthetic "organelles" of purple bacteria (Turner et al. 1973). Staining of dictyosomes appears to be more variable. In Zea mays root apices, Dauwalder and Whaley (1973) reported the proximal face of dictyosomes stained with ZIO in cells of the epidermis and rootcap, whereas in cortical parenchyma of Euphorbia characias both distal and proximal faces of dictyosomes stained, while several internal cisternae remained unstained (Marty, 1973b). Marty (1973a, b) concluded that dictyosomes play a role in the production of vacuoles in various cells in addition to their known secretory function.

The osmium tetroxide technique when applied to poppy laticifers stained both endoplasmic reticulum and vesicles. Adjacent cells showed staining of endoplasmic reticulum and the proximal face of dictyosomes.

In other plant tissue, impregnation with osmium tetroxide differentially stained the nuclear envelope (Dauwalder and Whaley, 1973; Chardard, 1973; Noguchi, 1976), vacuoles (Chardard, 1973; Noguchi, 1976), tannin vacuoles (Poux, 1973), internal pro-plastid membranes (Dauwalder and Whaley, 1973; Noguchi, 1976; Poux, 1973), mitochondria (Noguchi, 1976) and the proximal face of dictyosomes (Micalef, 1975; Noguchi, 1976; Poux, 1973).

Results derived from the osmium tetroxide impregnation procedure coupled with staining patterns previously reported for this technique support the hypothesis that laticifer vesicles are derived from dilation of endoplasmic reticulum.

Dickenson and Fairbairn (1975) described electron-dense regions or caps associated with alkaloidal vesicles and proposed that capped vesicles represented the mature form of previously "granulated" non-capped vesicles. Reports from earlier investigations are contradictory on this point in that caps have been described as occurring in young laticifers (Schulz, Schnepf and Mothes, 1967) and also fully differentiated cells (Thureson-Klein, 1970).

The developmental sequence described in the present study indicates that capped vesicles are most abundant and most conspicuous in intermediate states of laticifer differentiation. Capped vesicles appear to represent a transitional form between the small capless vesicles of immature laticifer elements and the large irregularly shaped vesicles characteristic of mature cells. The dense packing of enlarged capped vesicles at maturity obscures the caps and probably contributes to their granular appearance.

CONCLUSIONS

Development of alkaloidal vesicles in laticifers of opium poppy, Papaver somniferum L. was investigated at the ultrastructural level. Laticifer initials possessed abundant endoplasmic reticulum throughout their dense cytoplasm. During differentiation, the endoplasmic reticulum organized into long folded sheets that were parallel to the longitudinal walls along the periphery of the cell. Vesicles appeared to be derived from dilation of endoplasmic reticulum. This relationship was confirmed through cytochemical data obtained with zinc iodide-osmium tetroxide and osmium tetroxide impregnation. Alkaloidal vesicles had electron-dense regions or caps that occurred early in laticifer differentiation, but these caps became less conspicuous in mature cells. Caps appeared to be derived from small electron-dense and electron transparent particles which condensed along the inner surface of the vesicle membrane and subsequently accumulated at one or two positions along the membrane of the vesicle.

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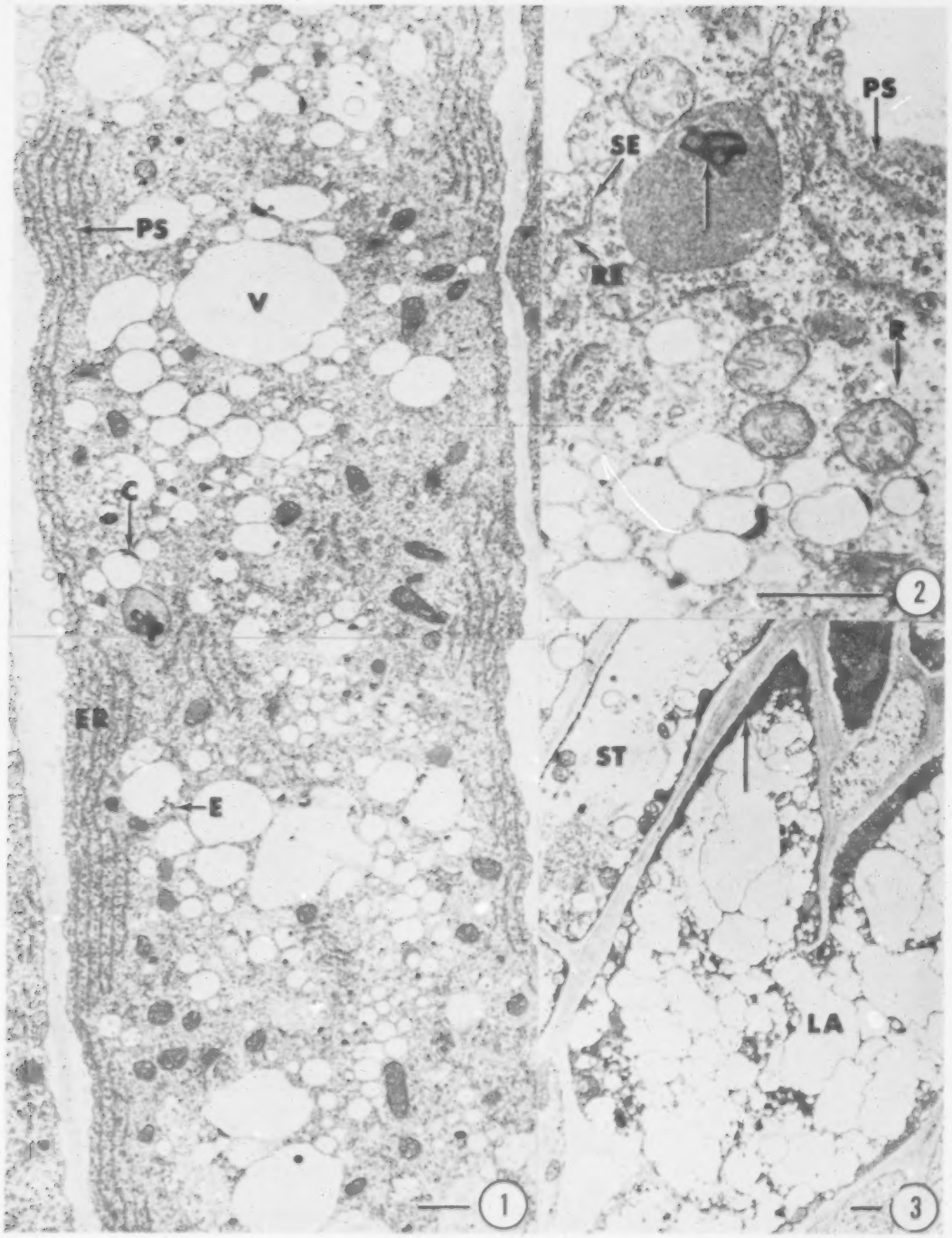
Figures 1 - 23

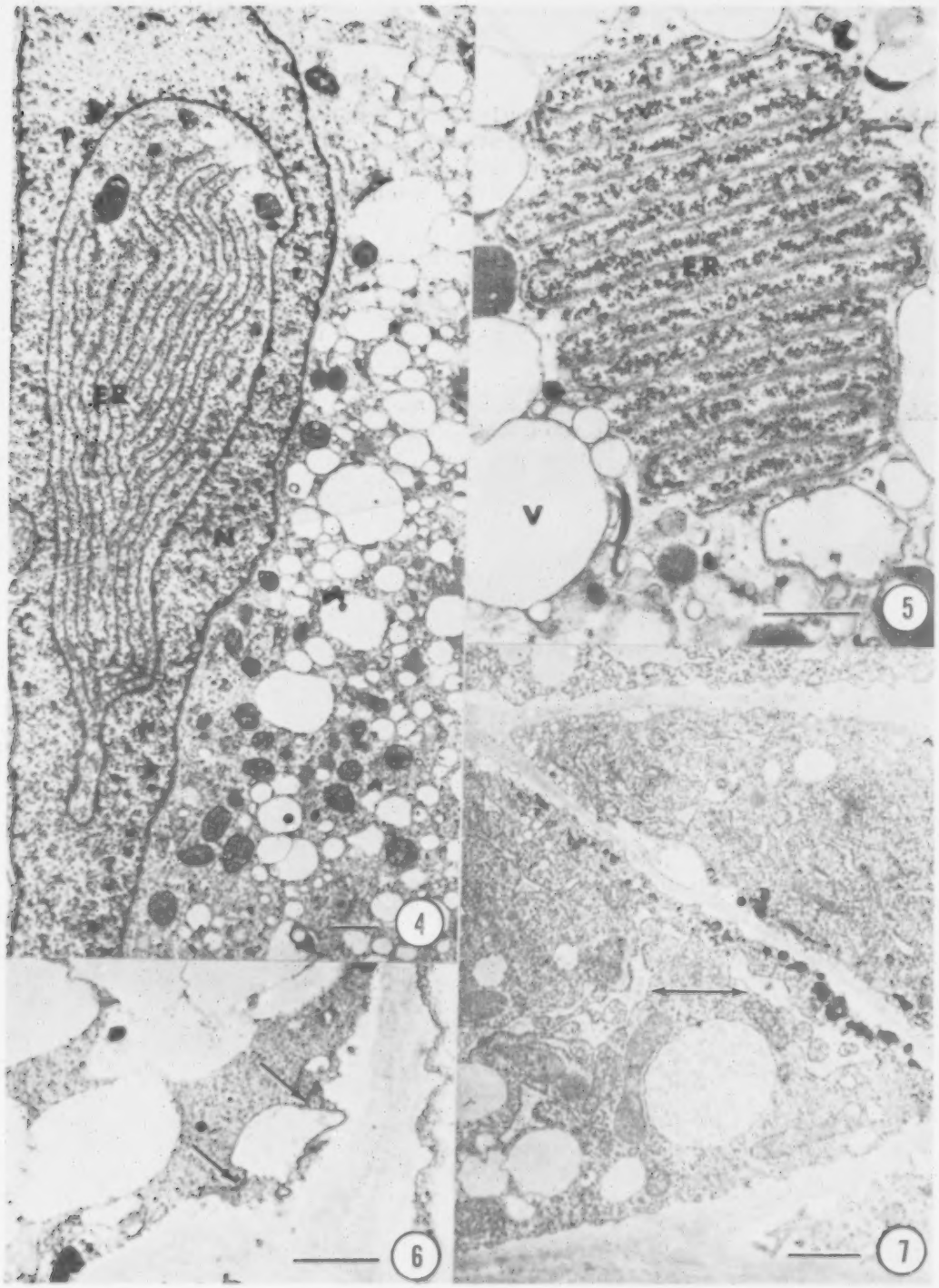


Figures 1-2: Laticifers in P. somniferum.
Fixation: Karnovsky/OsO_4. Magnification line represents 1 µm.

Figure 1 - Laticifers in early vesiculation stage of development from 50 hr radicle showing polysomes (PS) concentrated in regions of stacked endoplasmic reticulum (ER). C: cap; E: electron dense particles; P: plastid; V: vacuole.

Figure 2 - Laticifers in early vesiculation stage of development from 35 hr radicle showing polysomes (PS) and smooth endoplasmic reticulum (SE) alternating with rough endoplasmic reticulum (RE). Arrow: plastid core; R: ribosomes.

Figure 3 - Mature laticifer (LA) from leaf midvein adjacent to sieve-tube member (ST). Arrow: peripheral cytoplasm.



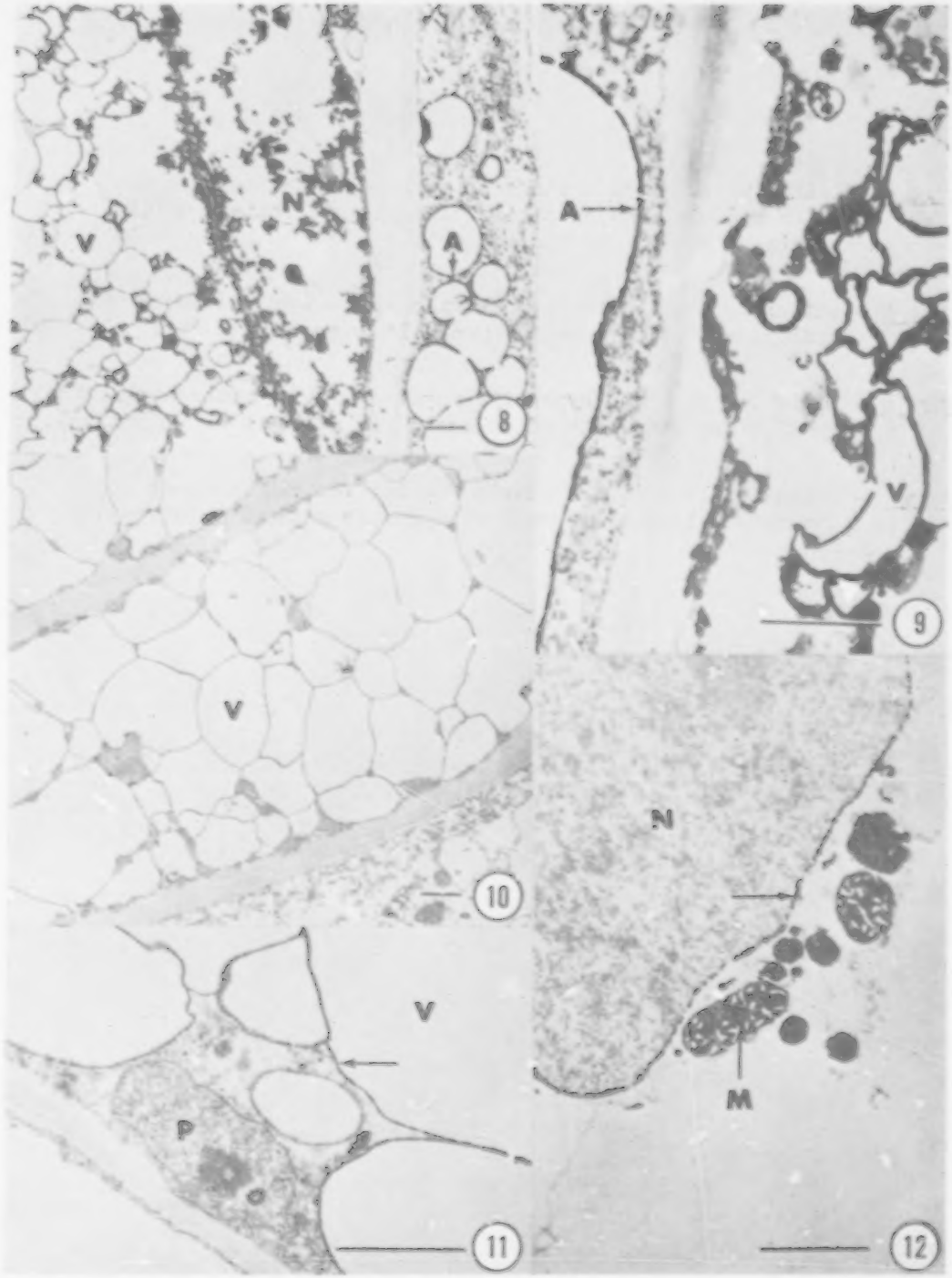
Figures 4-7: Laticifers in P. somniferum. Fixation: Fig. 4, 6, 7 Karnovsky/OsO_4; Fig. 5, 2% glutaraldehyde/OsO_4. Magnification line represents 1 µm in Fig. 4, 6, 7 and 0.5 µm in Fig. 5.

Figure 4 - Laticifer in early vesiculation stage of development from 50 hr radicle showing stacked endoplasmic reticulum (ER) within a lobe of the nucleus (N).

Figure 5 - Laticifer from shoot apex showing stacked endoplasmic reticulum (ER) surrounded by developing vesicles (V).

Figure 6 - Laticifer from the hypocotyl region showing vesicle formation by dilation of smooth endoplasmic reticulum (between arrows).

Figure 7 - Laticifer from 90 hr radicle showing vesicle formation by apparent dilation of rough endoplasmic reticulum (arrow).



Figures 8-12: Tissues from P. somniferum impregnated with zinc iodide-osmium tetroxide (ZIO). Magnification line represents 1 µm.

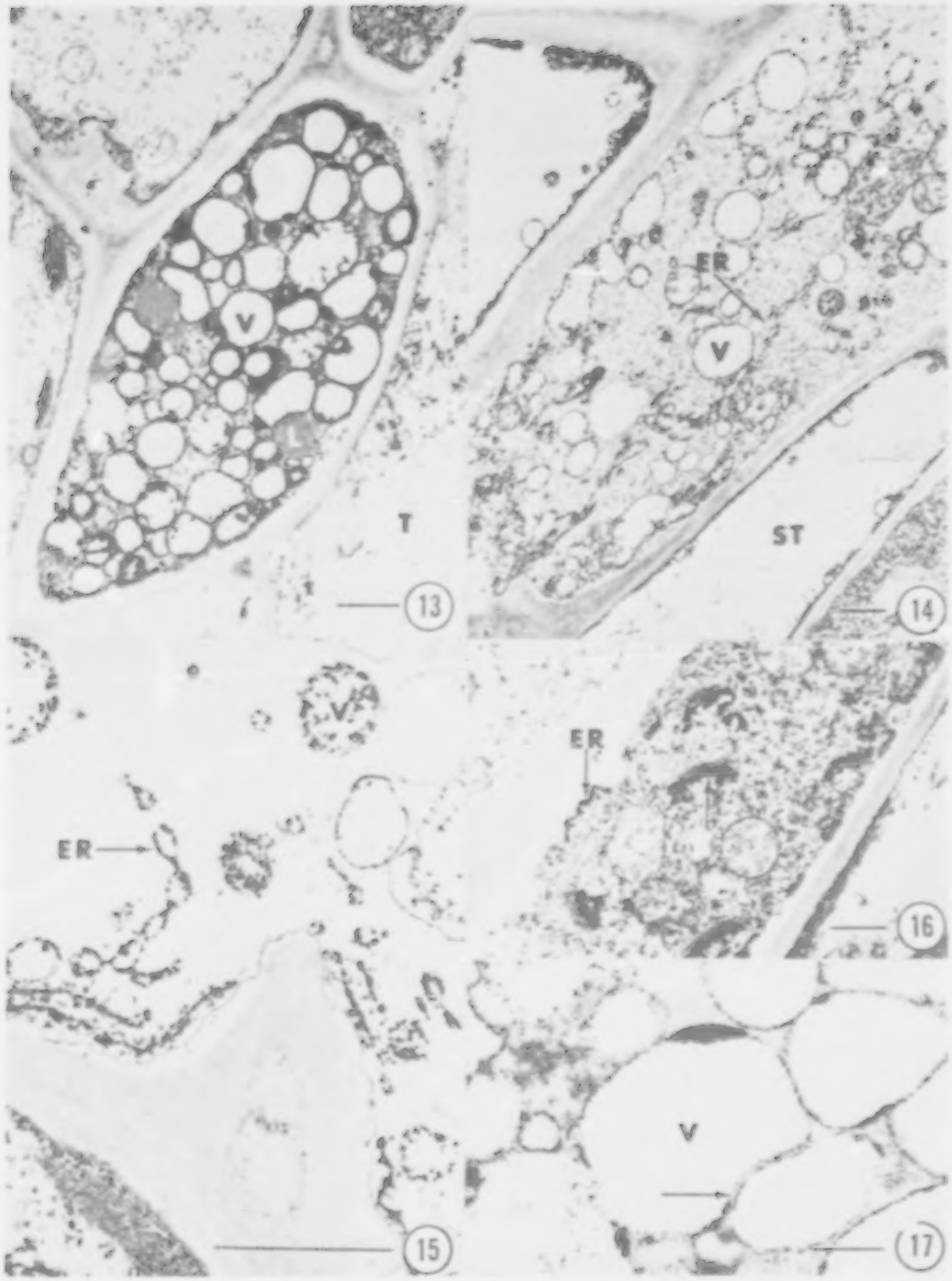
Figure 8 - Mature laticifer from leaf midvein with stained vesicles (V) and adjacent parenchyma with stained tonoplast (A) after ZIO impregnation. N: nucleus.

Figure 9 - Mature laticifer from shoot apex with stained vesicles (V) and adjacent parenchyma with stained tonoplast (A) after ZIO impregnation.

Figure 10- Laticifers of the petal with stained vesicles (V) after fixation in Turner's fixative followed by ZIO impregnation.

Figure 11- Laticifers of the petal showing differential staining of vesicular membranes (arrow) after fixation in Turner's fixative followed by ZIO impregnation. P: plastid; V: vesicle.

Figure 12- Non-laticiferous cell from 96 hr radicle showing differential staining of the perinuclear space (arrow) and mitochondrial matrices (M) after fixation in Turner's fixative followed by ZIO impregnation. N: nucleus.



Figures 13-17: Tissues from P. somniferum impregnated with osmium tetroxide. Magnification line represents 1 µm in Fig. 13-15, 2 µm in Fig. 16 and 0.5 µm in Fig. 17.

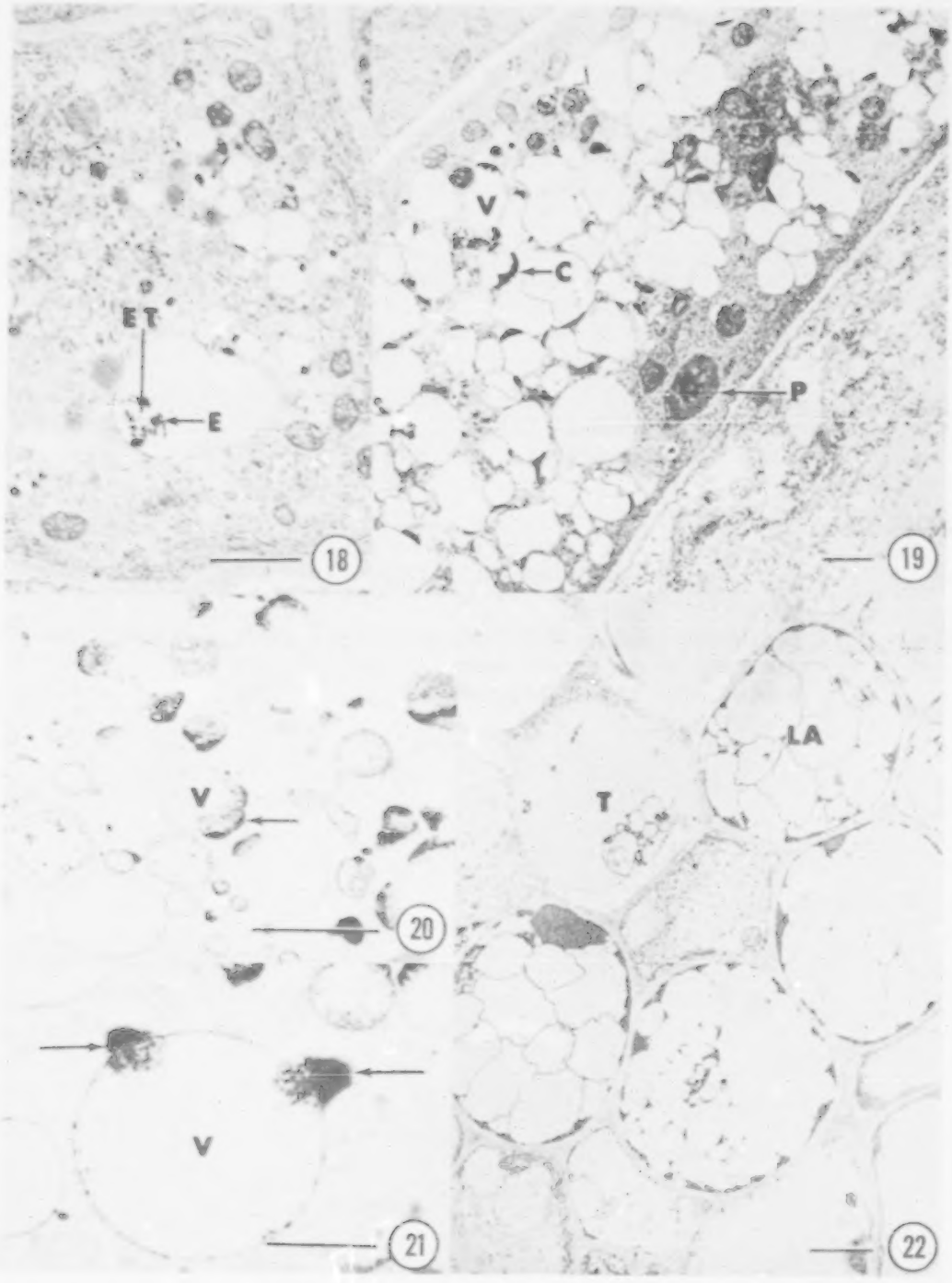
Figure 13 - Mature laticifer from first seedling leaves showing differential staining of vesicles (V) after osmium tetroxide impregnation. L: lipid droplet; T: tracheary element.

Figure 14 - Laticifer in early stage of vesiculation from first seedling leaves showing differential staining of vesicles (V) and dilating endoplasmic reticulum (ER) after osmium tetroxide impregnation. ST. sieve-tube member.

Figure 15 - Mature laticifer from leaf midvein showing differential staining of vesicle (V) and dilating endoplasmic reticulum (ER) after osmium tetroxide impregnation.

Figure 16 - Non-laticiferous cell from leaf midvein showing differential staining of the forming face of dictyosomes (arrow) and endoplasmic reticulum (ER) after osmium tetroxide impregnation.

Figure 17 - Laticifer vesicle (V) from shoot apex showing differential staining along the interior of its membrane (arrow) after osmium tetroxide impregnation.



Figures 18-22: Laticifers in P. somniferum. Fixation: Fig. 18, 19 Karnovsky/OsO_4; Fig. 20, 21 OsO_4; Fig. 22 Turner/OsO_4. Magnification line represents 1 µm in Fig. 18-20, 0.5 µm in Fig. 21 and 2 µm in Fig. 22.

Figure 18 - Laticifer in early stage of vesicle formation from 35 hr radicle showing the accumulation of electron-dense (E) and electron-transparent (ET) particles into the vesicle cap.

Figure 19 - Laticifer in late stage of vesicle formation from 100 hr radicle showing the abundant electron-dense caps (C) at the periphery of vesicles (V). P: plastid.

Figure 20 - Laticifer vesicles (V) with granulated cap (arrow) in seedling midvein fixed in osmium tetroxide.

Figure 21 - Laticifer vesicle (V) with two granulated caps (arrows) in seedling midvein fixed in osmium tetroxide.

Figure 22 - Cross section through a vascular bundle of the petal showing four latex vessels (LA) and an adjacent tracheary element (T).

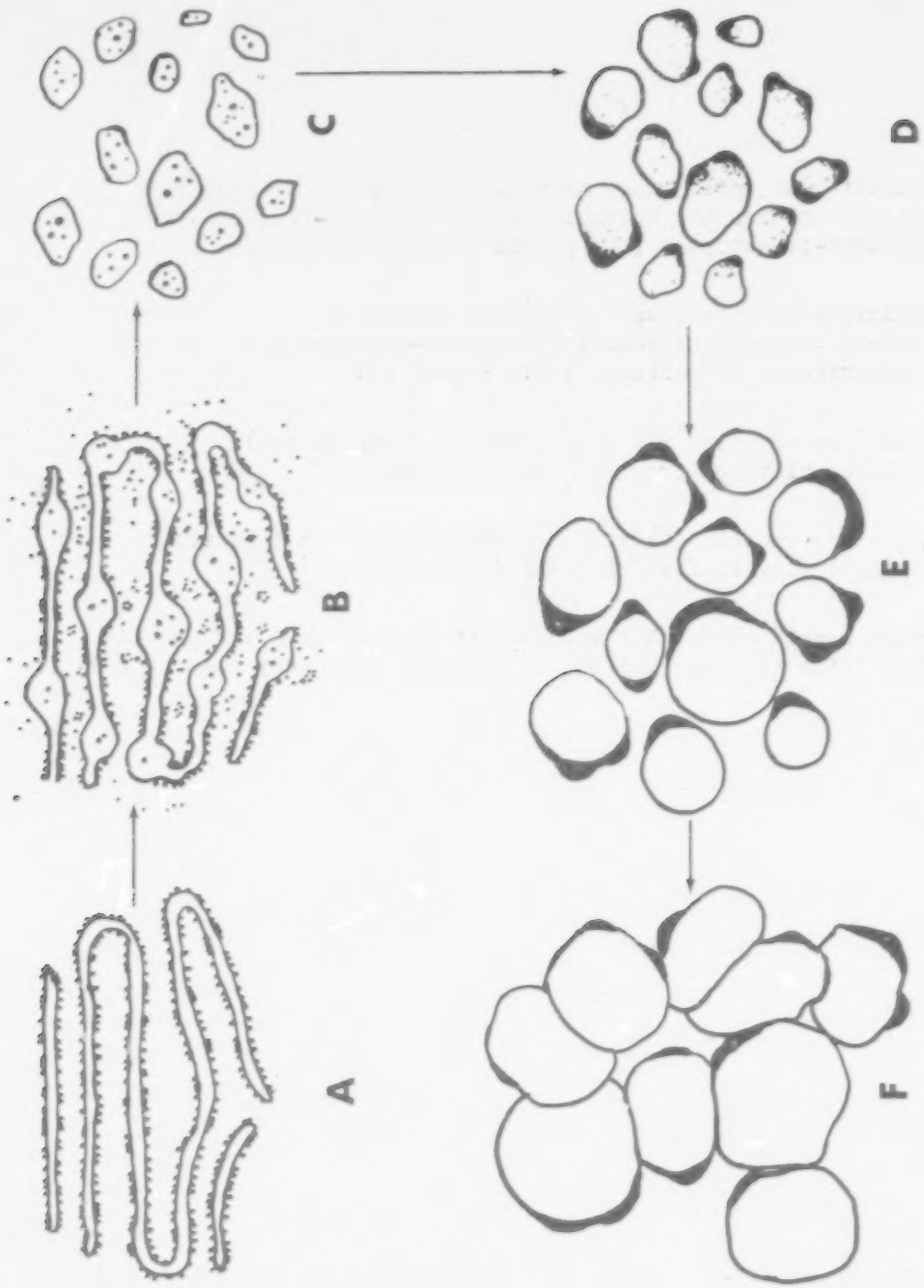


Fig. 23

Fig. 23 - Diagram of vesicle formation from dilation of endoplasmic reticulum. A. Stacked or unstacked endoplasmic reticulum. B. Dilation of endoplasmic reticulum with ribosomes. C. Small vesicles with electron-dense and electron-transparent particles. D. Concentration of particles along surface of enlarging vesicles. E. Capped vesicles, before complete vesicle expansion. F. Large irregularly shaped vesicles in mature cell. Caps obscured by the dense packing of vesicles.

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SCIENTIFIC RESEARCH ON PAPAVER SPECIES AS SOURCES OF CODEINE, MORPHINE AND THEBAINE 1/

No. 26

REPORT ON RESEARCH IN EGYPT TO SELECT PAPAVER SOMNIFERUM PLANTS WITH A HIGH MORPHINE CONTENT

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1/ This ST/SOA/SER.J/.. series of documents was previously entitled "Scientific research on Papaver bracteatum".

2/ Professor G.H. Mahran and Dr. I. Salad Ahmed, Faculty of Pharmacy, Cairo University, collaborated in this research project.

General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

E. 77-1511

INTRODUCTION

Progress in medical and related health services throughout the world has increased the demand for codeine, a widely used antitussive and mild analgesic. To meet this expanding demand, adequate supplies of this drug must be ensured. Therefore, new technology is required to increase the yield per unit area of phenanthrene alkaloids.

The purpose of this research is (1) to identify Papaver somniferum plants with a high morphine content which could be used in other countries to develop superior varieties and (2) to determine the number of successive seed crops that can be grown in Egypt in a 12 months period.

METHOD AND MATERIALS

Experiments were conducted in collaboration with the Faculties of Pharmacy at the Universities of Alexandria, Asyût and Cairo, representing a north to south distance of 570 km. Alexandria has a Mediterranean climate while Cairo and Asyût have desert climates.

Papaver somniferum seed from the following six sources was provided by the U.N. Narcotics Laboratory and it was used in the experiments at each location:

UNS 1	P. somniferum	seed	from	Hungary
UNS 2	"	"	"	Norway
UNS 3	"	"	"	Switzerland
UNS 4	"	"	"	Hungary
UNS 13	"	"	"	Spain
UNS 39	"	"	"	Turkey.

At Alexandria the soil was a loam; at Cairo and Asyût a clay/loam. Chemical and mechanical soil analyses for the experimental site at Cairo are given in Table 1. At the three locations, the experiments consisted of six square plots. Each plot was divided into six lines (planting beds) 2.5 m in length and 70 cm between lines. Two rows spaced 20 cm apart were sown on each

line. The seeds were mixed with sand and an aliquot with 8 to 10 seeds was sown in shallow pits 10 cm in length and covered with a thin layer of soil. The soil was irrigated to maintain the required level of moisture for germination and plant growth.

The dates of sowing the experiments were:

Alexandria	17/10/75
"	12/5/76
Cairo	9/4/75
"	10/11/75 */
"	26/1/76
"	17/3/76
"	25/5/76
Asyût	20/1/76
"	15/3/76
"	20/5/76

*/ Initial sowing made on 14/10/75; resown due to poor seedling emergence.

After seedling emergence the plants were thinned to two per pit, spaced approximately 50 cm apart within the row. At the time of thinning, excess vigorous seedlings were transplanted to other plots arranged in the same way as those for the initial sowings.

After the young plants were making good growth, all plots were fertilized with $(NH_4)_2SO_4$ and K_2SO_4 at the rate of 50 kg of each per feddan (4,200 m^2). Weeds were controlled by hand cultivation.

At maturity, the capsules from each plant were harvested when pale straw to yellowish green in colour, placed in a separate container, assigned an identification number and air-dried. Later, the capsules were cut transversely; the seed collected and given the plant capsule identification number. The weight of the capsules without seed was recorded for each plant.

The following thin-layer chromatography procedure was used to quantify the morphine content of harvested capsules :

1. One gramme, weighed accurately, of each sample was introduced in an extraction flask, macerated overnight with 6 ml of solvent mixture (chloroform:isopropanol:ethanol:ammonium hydroxide in ratios 6:2:1.5:0.5 by volume). Flasks were shaken and extracts were filtered in 10 ml measuring flasks. Extraction was completed and volume adjusted to 10 ml.
2. Extracts were spotted on plates of silica gel 0.25 mm thickness along with spots of standard morphine solutions corresponding to 3,5 and 7 µg morphine.
3. Plates were developed with a solvent system acetone:benzene:methanol: ammonium hydroxide in ratios 99:83:10:8 by volume.
4. The developed chromato-plates were sprayed with 1% aqueous picric acid, dried at 60-70°C then sprayed with 2% aqueous iodine.
5. Plates were examined under U.V. (254 mµ).

The weighted average percent morphine content for each UNS number was calculated from the weight of capsules and morphine yield per plant.

RESULTS AND DISCUSSION

Cairo

Seed of the six P. somniferum selections sown on 10 November 1975 gave useful observations on time of sowing, plant growth and development and morphine content in the capsules of individual plants. A summary of observations on several plant characteristics under study is given in Table 2.

The number of plants in each seed source ranged from 93 to 143. Generally, the plants grew satisfactorily and many reached a maximum height at maturity in the range of 100 to 155 cm except UNS-13 which was only 35 cm. Scorching symptoms on the leaves was generally evident among the plants; UNS-1 and UNS-4 showed the least damage. All plants were attacked by a fungus at the time of flowering which hindered capsule development.

The best plant growth and flower development occurred in UNS-4 followed by UNS-1 and UNS-39. Although growth was slower in UNS-2 and UNS-3, it was by comparison much poorer in UNS-13. Either the latter ecotype is morphologically different from the others or the local environmental pressures restricted plant growth, flowering and capsule size.

UNS-4 was the earliest flowering seed source. The first flower was noted on 26 February. UNS-13 was latest to flower, producing the first flower on 5 April.

Of significant importance was the variation among seed sources in the proportion of plants producing mature capsules. In UNS-1 and UNS-4 79% and 69% respectively of the plants produced usable capsules. The proportion of capsule bearing plants for the other seed sources was much lower: 38% for UNS-3; 37% for UNS-39; 31% for UNS-13 and 30% for UNS-2. Thus the total yield of capsules per unit area was significantly reduced for the latter four seed sources.

The average number of capsules per plant ranged from 5.8 for UNS-39 to 2.4 for UNS-2. One plant in the UNS-39 population produced 42 capsules. The average weight of capsules per plant varied from 5.1 g for UNS-4 to 0.8 g for UNS-13. The latter had very small capsules measuring 1.5 cm in length by 1 cm in width.

The morphine content for the different seed sources was 0.58% in UNS-13; 0.55% in UNS-39; 0.46% in UNS-2; 0.44% in UNS-4 and 0.39% in UNS-1. The percentages of morphine fall generally within the normal range for straw. However, the morphine content of UNS-13 is much lower than that found in capsules grown in Spain.

In identifying plants for further investigation, the quantity and concentration of morphine were the primary selection criteria. Table 3 shows the distribution of plants within each UNS seed source according to the quantity of morphine they produced. Plants in the populations of UNS-1, UNS-4 and UNS-39 yielded more than 6.99 mg of morphine. UNS-4 had the most plants in the higher yield classes. After one or two additional cycles of evaluation,

seed of plants producing the largest quantity of morphine will be forwarded to countries breeding new varieties of P. somniferum with higher yield of phenanthrene alkaloids per unit area.

Table 5 also gives the distribution of plants within each UNS number according to the percent of morphine. Plants with a morphine content of 0.7% or higher were found in each of the UNS seed sources. Of the 344 individual plants analysed by the TLC method in this experiment, 60 or 17% had capsules with a morphine concentration of 0.7% or better. The high morphine plant selections will be included in the next evaluation test at Cairo. In addition seed from a number of these plants will be sown at Asyût to obtain information on the effect of environment on morphine content.

Experiments sown at Cairo on 9 April 1975, 26 January 1976, 17 March 1976 and 25 May 1976 failed to produce usable plant materials. Seeds sown on 9 April germinated and produced seedling plants. However, the high temperatures which prevail at that time of year together with the Khamasin winds caused most of the plants to wither and die. Climatic conditions were unfavourable for the sowing made on 26 January. No plants grew to the flowering stage. Seed germination was poor following the 17 March and 25 May sowings. Temperatures were too high and the seedling plants which did emerge were weak and soon died. Thus results from the date of sowing research show the best time to sow P. somniferum seed is from early October to mid-November. This period will provide the maximum germination, seedling emergence and plant growth. Sowings made at other dates encounter unfavourable weather conditions for germination and the plants which emerge wither and die or fail to make normal growth, flower or produce mature capsules.

Generally, seedlings transplanted at the time of thinning did not survive. Only a few trans-plants of UNS-5 and UNS-39 produced mature capsules. They had a lower average morphine content probably caused by the delay in growth due to the "shock" of trans-planting.

Alexandria

Neither of the experimental sowings were successful at this location because of unfavourable climatic conditions. It can be concluded that the

propagation of P. somniferum germplasm is not practical under the specific conditions at this site.

Asyût

Sowings of P. somniferum seed on 20 January 1976, 15 March 1976 and 25 May 1976 were not successful. Although seed germination and plant growth was satisfactory following the earlier sowing, burning symptoms and fungus attacks on the leaves hindered flowering and capsule maturation. In the entire experiment, only six plants of UNS-4 and two plants of UNS-39 had usable capsules.

Unfavourable climatic conditions, particularly increasing temperatures, following the sowings on 15 March and 25 May caused the seedling plants to wither and die. In the March sowing 3 plants of UNS-4 and two plants of UNS-39 persisted for about one month then faded and died also. In the sowing made in May none of the seed germinated due to high soil temperatures.

New experiments will be sown in late October 1976.

CONCLUSIONS

1. Sixty individual plants of P. somniferum in the first cycle of evaluation were identified having capsules with a morphine content of 0.7% or greater as determined by HLC analyses.

2. a) At Cairo, UNS-4 and UNS-39 gave the largest percentages of high morphine yielding plants (mg morphine per plant);
   b) UNS-4 and UNS-39 had the largest number and weight of capsules per plant;
   c) There were significant differences among the six UNS seed sources in percentage of plants producing mature capsules;
   d) UNS-13 followed by UNS-39 had the highest average morphine percentage;
   e) UNS-39 had the largest percentage of plants with a morphine content of 0.7% or greater.

3. Fall sowing (mid-October to mid-November) of poppy seed at Cairo provides the best climatic conditions for germination, seedling emergence and normal plant growth and development. Sowings in January, March, April and May have been failures due to unfavourable climate and diseases.

4. Seed sown at Alexandria in October and May, and at Asyût in January, March and May failed to develop into usable plant materials.

5. The research site near Alexandria is unsuitable for the experiments with the opium poppy.

6. Transplanting seedlings from fall sowings at Cairo resulted in a high plant mortality, reduced growth and a lower morphine content.

7. Seed from each of the 60 selected plants will be sown in a second cycle evaluation test in October 1976.

Table 1

Mechanical and chemical analyses of the soil in the experimental field at Cairo, Egypt

Mechanical analysis:

Coarse sand	4.10%
Fine sand	34.22%
Silt	27.70%
Clay	22.85%
$Ca\ CO_3$	3.07%
Texture	Clay loam

Chemical analysis:

Total soluble salts	0.35%
$H\ CO_3$	1.20%
Cl	1.56%
SO_4	1.70%
Ca	1.60%
Mg	0.85%
Na	2.50%
Available N	29.20 ppm
Available P	20.70 ppm
Available K	755.00 ppm
pH	7.9

Table 2

Observations on Papaver somniferum plants from different seed sources at Cairo University 1/

	Seed sources from United Nations Laboratory					
	UNS-1	UNS-2	UNS-3	UNS-4	UNS-13	UNS-39
PLANTS						
Date of emergence	23/11/75	25/11/75	25/11/75	22/11/75	23/11/75	24/11/75
No. after thinning	101	120	101	143	98	93
Height at maturity (cm)	120 - 155	65 - 130	60 - 100	90 - 145	30 - 35	80 - 110
Stem diameter (cm)	5	5	4	4	2	6
No. with mature capsules	80	36	38	99	30	34
LEAVES						
Length (cm)	40 - 55	50 - 58	50 - 58	40 - 60	30 - 37	40 - 55
Breadth (cm)	18 - 21	17 - 22	22 - 28	18 - 25	16 - 20	14 - 22
FLOWERS						
Date of first flowering	10/3/76	25/3/76	28/3/76	26/2/76	5/4/76	1/3/76
No. of flowering plants						
at 3/4	45	3	2	55	--	40
at 6/4	56	11	5	65	2	60
at 10/4	60	20	15	70	12	80
at 12/4	70	40	25	90	25	80
Colour of petals	White/Crimson base	White/pale rose base	White/Crimson base	White/Pale violet base	White/red base	Deep/Violet to Crimson base
No. of stigmas/flower	13 - 14	12 - 15	12 - 14	13 - 14	12 - 14	11-- 12
CAPSULES						
No. at 3/4	61	4	--	189	--	47
No. at 12/4	290	62	69	417	20	194
Total No.	327	86	129	485	109	199
Size: Length (cm)	5	3	3	5	1.5	4
Breadth (cm)	3	3	3	4	1.0	4
Average number per plant	4.1	2.4	3.4	4.9	3.6	5.8
Average wt/plant	4.1	1.6	1.6	5.1	0.8	4.5
Morphine content % 2/	0.39	0.46	0.43	0.44	0.58	0.55

1/ Date of sowing: 10.11.75; Date of thinning: 26.12.75; Date of harvest: 18.4.76.

2/ Weighted average morphine content of individual plant capsules in each UNS seed source.

Table 3

Frequency distributions of the quantity and concentration of morphine in P. somniferum produced from different seed sources at Cairo University

	Seed sources from United Nations Laboratory											
	UNS-1		UNS-2		UNS-3		UNS-4		UNS-13		UNS-39	
No. of plants	80		34		38		99		30		34	
Quantity of morphine	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Less than 1 mg	2	2.50	2	5.88	2	5.26	-	-	11	36.66	-	-
1-1.99 mg	9	11.25	10	29.41	22	57.89	5	5.05	14	46.66	2	5.88
2-2.99 mg	19	23.75	4	11.76	8	21.05	18	18.18	4	13.33	8	23.52
3-3.99 mg	19	23.75	7	20.58	4	10.52	21	21.21	-	-	6	17.64
4-4.99 mg	13	16.25	7	20.58	1	2.63	20	20.20	1	3.33	4	11.76
5-5.99 mg	6	7.50	3	8.82	1	2.63	14	14.14	-	-	7	20.58
6-6.99 mg	7	8.75	1	2.94	-	-	8	8.08	-	-	3	8.82
7-7.99 mg	3	3.75	-	-	-	-	7	7.07	-	-	1	2.94
8-8.99 mg	-	-	-	-	-	-	5	3.03	-	-	2	5.88
9-9.99 mg	1	1.25	-	-	-	-	2	2.02	-	-	1	2.94
More than 10 mg	1	1.25	-	-	-	-	1	1.01	-	-	-	-
Total %		100 %		99.97		99.98		99.99		99.98		99.96
Concentration of morphine												
Less than 0.3 %	14	17.50	1	2.94	-	-	3	3.03	-	-	-	-
0.3 %	35	43.75	14	41.17	21	55.26	38	38.38	-	-	3	8.82
0.4 %	1	1.25	-	-	-	-	-	-	-	-	-	-
0.5 %	21	26.25	12	35.29	12	31.58	39	39.39	22	73.33	19	55.88
0.6 %	1	1.25	-	-	-	-	-	-	-	-	-	-
0.7 %	8	10.0	4	11.76	5	13.15	17	17.17	3	10.0	11	32.00
More than 0.7 %	-	-	3	8.82	-	-	2	2.02	5	16.66	1	2.94

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SCIENTIFIC RESEARCH ON PAPAVER SPECIES AS SOURCES OF CODEINE, MORPHINE AND THEBAINE [1]

No. 27

HIGH PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION AND QUANTITATION OF THEBAINE FROM PAPAVER BRACTEATUM [2,3]

by: Fen-Fen Wu and R.H. Dobberstein [4]

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1/ This ST/SOA/SER.J/.. series of documents was previously entitled "Scientific research on Papaver bracteatum".

2/ In the report of the Second Working Group on P. bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in P. bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This research was supported by S.B. Penick and Company, Orange, New Jersey.

4/ This paper was submitted by Dr. Bernard B. Brown, Vice President of Research, S.B. Penick and Company.

General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

GE. 77-1513

Gas chromatography has been used routinely to separate and quantitate the thebaine in Papaver bracteatum [5-10]. However, none of these GC procedures has established that the thebaine peak represents pure thebaine. For this reason, a total alkaloid extract of P. bracteatum was injected on a GC column [11] and the compounds represented by the two resulting peaks were collected. GC operating conditions were: injector and detector temperature, 285°C; oven temperature, 270°C; flow rate of carrier gas (helium), 48 ml/min.; hydrogen pressure, 17.5 p.s.i.g. The two fractions obtained were each dissolved in chloroform and the samples were applied to a precoated silica gel GF_{254} thin-layer plate (0.25 mm) [12]. After developing with toluene-acetone-ethanol-conc. ammonium hydroxide (20:20:3:1) [13], the chromatogram was examined under both long and short wave UV light and sprayed with sulfuric acid [14] followed by charring with heat. Thebaine was apparently decomposed by the high temperature used

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5/ P. Cheng and N.J. Doorenbos, United Nations document ST/SOA/SER.J/5 (1973).

6/ F.J.E.M. Kuppers, R.J.J. Ch. Lousberg, C.A.L. Bercht and C.A. Salemink, United Nations document ST/SOA/SER.J/8 (1974).

7/ P.G. Vincent and W.A. Gentner, United Nations document ST/SOA/SER.J/9 (1974).

8/ M.J. Duffy, P.A. Ackert and C.F. Hiskey, United Nations document ST/SOA/SER.J/18 (1975).

9/ D. Furmanec, J. Chromatogr. 89, 76 (1974).

10/ C.B. Coffman, C.E. Bare, and W.A. Gentner, Bull. Narcot., 27-3, 41 (1975).

11/ A Perkin-Elmer Model 881 gas chromatograph equipped with a hydrogen flame ionization detector and a Seargent SR recorder were used in this study. A spiral glass chromatographic column (6 ft. long, 1/4 in. O.D.) was packed with 2.5% OV-17 on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories Inc. State College, Pennsylvania).

12/ E. Merck, Darmstadt, Federal Republic of Germany.

13/ E. Stahl, H. Jork, E. Dumont, H. Bohrmann and H. Volmann, Arzneimittel. Forsch., 19, 194 (1969).

14/ Concentrated sulfuric acid dissolved in methanol (70% v/v).

during gas chromatography, since no spot corresponding to thebaine could be observed. The two collected fractions were qualitatively identical, containing at least four compounds but were quantitatively different. It was also observed that the ratios of one compound to another in each fraction was dependent on the column temperature employed. Since thebaine is decomposed under the conditions used in GC analysis, it is impossible to determine whether the thebaine peak represents pure thebaine or a mixture of phyto-constituents and/or their decomposition products. For this reason, a high pressure liquid chromatographic procedure employing milder conditions was developed.

A number of high pressure liquid chromatographic separations of thebaine and other opium alkaloids have been previously reported [15-22]. However, most of these methods require either pre-treatment of the extract to remove polar impurities (e.g., ion-exchange chromatography) or gradient elution equipment, and/or they do not produce base line separation of thebaine

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15/ D.W. Smith, T.H. Beasley, Sr., R.L. Charles, and H.W. Ziegler, J. pharm. Sci., 62, 1691 (1973).

16/ I. Jane, J. Chromatogr., 111, 227 (1975).

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19/ C-Y. Wu, S. Siggia, T. Robinson and R.D. Waskiewicz, Anal. Chim. Acta, 63, 393 (1973).

20/ J.H. Knox and J. Jurand, J. Chromatogr., 82, 398 (1973).

21/ I. Jane and J.F. Taylor, J. Chromatogr., 109, 37 (1975).

22/ P.J. Twitchett, J. Chromatogr., 104, 205 (1975).

from other P. bracteatum constituents. A rapid, single column separation which produces base line separation of thebaine, but which does not require prior removal of polar impurities or gradient elution equipment was sought.

Preparation of plant extracts for chromatography

Dried, 40-mesh P. bracteatum (1.0 g) was transferred to a 125 ml Erlenmeyer flask and 5% aqueous acetic acid (50 ml) added. The flask was shaken for one hour on a rotary shaker, the mixture was filtered through a Büchner funnel and the marc was washed with 5% aqueous acetic acid (10 ml). Following alkalinization of the filtrate with concentrated ammonium hydroxide (6 ml), it was extracted three times with chloroform (50 ml each extraction). The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the sodium sulfate was washed with chloroform (10-15 ml). The combined extracts were evaporated to dryness in vacuo, yielding a residue which was suitable for direct application to a chromatographic column.

High pressure liquid chromatography [23/]

The residue from the chloroform extract was dissolved in an accurately measured volume of methanol-water (8:2), containing 0.3% ammonium carbonate and an aliquot was injected onto a μBondapak C_{18} column [24/] (1 ft. long, 3/8 in. O.D.). Operating conditions were: ambient temperature; flow rate of eluting solvent (methanol-water containing 0.3% ammonium carbonate, 8:2), 1 ml/min.; wavelength of UV detector, 285 nm. Under these conditions, the following retention times were obtained: thebaine, 10.2 min.; isothebaine, 6.0 min.; and orientalidine, 6.0 min. Although orientalidine and isothebaine are not resolved, a sharp, symmetrical peak with base line separation was obtained for thebaine, and the entire separation and quantitation procedure required only 12 minutes.

23/ These studies were conducted using a Waters Associates Model 6000 liquid chromatograph equipped with a Beckman 25 variable wavelength UV spectrophotometer and recorder.

24/ Waters Associates Inc., Milford, Massachusetts.

Although μBondapak C_{18} consists of a C_{18} alkane chemically bonded to silica *via* a hydrolytically stable ether linkage, repeated use of these columns appeared to give rise to a small number of active sites on the silica. It was therefore necessary to saturate these active sites by making a daily injection of 20 μg thebaine standard. In addition, injections of 5 μg thebaine standard were made until successive injections gave thebaine peak areas within two percent of each other or better [25].

In order to determine whether the thebaine peak represents only pure thebaine, twelve repeated injections of *P. bracteatum* straw extracts were made and the thebaine fractions were collected, combined, and evaporated to dryness *in vacuo* to yield approximately 150 μg of residue. This residue was dissolved in chloroform and the total sample was applied to a pre-coated silica gel GF_{254} thin-layer plate (0.25 mm) [12]. The chromatogram was developed a distance of 15 cm. from the point of sample application using toluene-acetone-ethanol-conc. ammonium hydroxide (20:20:3:1) [13]. The developed chromatogram was examined under both long and short wave UV light and then sprayed with sulfuric acid [14] and charred by heating. Only a single spot was observed, corresponding to thebaine (R_f 0.34) [26]. In a second experiment, six repeated injections of *P. bracteatum* straw extracts were made and the thebaine fractions were collected, combined, and evaporated to dryness in vacuo to yield 30 μg of residue. The UV spectrum of this residue dissolved in methanol was superimposable with that of thebaine standard. The purity of the collected thebaine sample was confirmed by thin-layer chromatography.

25/ Usually two injections of 5 μg each are sufficient.

26/ In this chromatographic system, isothebaine and orientalidine had R_f values of 0.40 and 0.52 respectively. Isothebaine and orientalidine were generously provided by Professor E. Brochmann-Hanssen, University of California, San Francisco.

Two standard Beer's law plots were obtained by injecting different concentrations [27] of standard thebaine on the column in quadruplicate and measuring the thebaine peak areas by the triangulation method. In both cases linear plots were obtained with reliabilities of 0.99943 and 0.99928.

Thebaine can therefore be rapidly separated from a P. bracteatum alkaloid extract and quantitated using a single high pressure liquid chromatographic system. This procedure also permits the collection of the thebaine peak and the analysis of this thebaine for purity using other techniques (TLC, UV, mass spectrometry).

27/ The concentrations used were: 0.2, 0.4, 1.0, 1.5 and 2.0 µg (0.25 absorbance units full scale on UV recorder) and 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 µg (0.5 absorbance units full scale on UV recorder).